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Regulation of Prostaglandin E_2 release in cerebral ischemia

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Prof. Dr. Markus Schwaninger

Dedicated to my family

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Contents

Summary

Arachidonic acid (AA) and its metabolites are implicated in the induction and/or resolution of inflammation. Prostaglandin E_2 (PGE_2) is a metabolite of AA that is known to have neurotoxic effects in several pathophysiological conditions such as ischemia. PGE_2 is produced as a result of the combined activities of several genes including, cytosolic phospholipase A-2 (cPLA-2), cyclooxygenase-2 (COX-2) and microsomal prostaglandin E2 synthase-1 (mPGES-1).

We have shown that the genes responsible for PGE_2 synthesis were upregulated following ischemia both *in vivo* in mice subjected to middle cerebral artery occlusion (MCAO) and *in vitro* in primary cortical neurons subjected to oxygen glucose deprivation (OGD).

We also provided *in vivo* and *in vitro* evidence that this upregulation was dependant on NF-κB signaling. *In vivo*, mice expressing an inhibitor of the NF-κB pathway in neurons showed an abolished upregulation of the AA cascade genes cPLA-2, COX-2 and mPGES-1 after MCAO. *In vitro,* reporter fusion genes in which the promoter sequence for each of the three AA cascade genes was inserted into a promoterless vector showed that the NF-κB activators TNF, constitutively active IKK2 or p65 stimulated the transcription of cPLA-2, COX-2 and mPGES-1 in primary neurons providing evidence for the involvement of NF-κB in the regulation of these genes.

High mobility group box 1 protein (HMGB1), a nuclear protein, was recently shown to have a cytokine like activity acting as a late mediator of inflammation in several models of inflammation by acting on one or more receptors including receptor for advanced glycation end products (RAGE) and Toll like receptors (TLR-2 and -4).

HMGB1 was shown in our study to have a role in mediating the toxic effect observed after OGD in primary cortical neurons and in mixed neural cultures containing neurons, astrocytes and microglia. Neurons released HMGB1 after OGD and blocking the effects of HMGB1 using the decoy receptor sRAGE was protective. However, stimulation with recombinant HMGB1 was only toxic to mixed

neural cultures and this effect was mediated through RAGE on microglia which responded to HMGB1 by the production of PGE_2 which further promotes neurotoxicity.

Zusammenfassung

Arachidonsäure (AS) und ihre Stoffwechselprodukte sind an der Induktion und/oder Beseitigung von Entzündungen beteiligt. Prostaglandin E_2 (PGE_2) ist ein Stoffwechselprodukt von AS, das für seine neurotoxische Wirkung in verschiedenen pathophysiologischen Prozessen bekannt ist. PGE_2 entsteht durch die Aktivität der zytosolischen Phospholipase A-2 (cPLA-2), der Cylcooxygenase-2 (COX-2) und der microsomale Prostaglandin E_2 Synthetase (mPGES-1).

In dieser Arbeit konnte gezeigt werden, dass die für die PGE_2-Synthese verantwortlichen Gene infolge einer Ischämie sowohl *in vivo* nach Okklusion der *Arteria cerebri media* der Maus (middle cerebral artery occlusion, MCAO), als auch *in vitro* nach Sauerstoff-Glukose-Deprivation (oxygen glucose deprivation, OGD) primärer cortikaler Neuronen, hochreguliert werden.

Außerdem wurden *in vivo* und *in vitro* Hinweise auf eine NF-κB-Abhängigkeit dieser Hochregulierung geliefert. *In vivo* zeigten Mäuse, die einen Inhibitor des NF-κB-Signawegs exprimieren, eine reduzierte Hochregulierung der Gene der Prostaglandin-Synthese cPLA-2, COX-2 und mPGES-1 nach MCAO. *In vitro* konnte durch Reporterfusionsgene, in welchen die Promotorsequenz jeweils eines der drei AS-Kaskadengene in einen Promotor-losen Vektor eingefügt wurden, gezeigt werden, dass der NF-κB-Aktivator TNF, oder Überexpression der konstitutiv aktive IKK2 oder p65 die Transkription von mPGES-1, cPLA-2 und COX-2 in primären corticalen Neuronen stimulieren, was auf eine Beteiligung von NF-κB an der Regulation dieser Gene hinweist.

Das *High Mobility Group Box 1 Protein* (HMGB1) ist ein nukleäres Protein, dessen Cytokin-ähnliche Aktivität in verschiedenen Entzündungsmodellen als später Entzündungsmediator mit Einfluss auf einen oder mehrere Rezeptoren wie *Receptor for Advanced Glycation End Product* (RAGE) und *Toll-like Receptor* (TL-2 und TL-4), kürzlich entdeckt wurde.

Die vorliegende Studie zeigt, dass HMGB1 eine Rolle in der Vermittlung toxischer Effekte der OGD in primären corticalen Neuronen und in gemischten Kulturen aus Neuronen, Astrozyten und Mikroglia spielt. Neuronen setzten HMGB1 nach OGD frei, während eine Blockierung des HMGB1-Effekts unter Verwendung des löslicher Rezeptors sRAGE protektiv wirkte. Die Stimulation mit rekombinantem HMGB1 hatte nur eine toxische Wirkung auf gemischte Kulturen. Dieser Effekt wurde durch die Wirkung von RAGE auf Mikroglia vermittelt, die auf HMGB1 mit der Produktion von PGE_2 reagierten, was die Neurotoxizität weiter verstärkte.

2. Introduction

2.1. Definition and impact of stroke

Stroke is the third leading cause of death and the leading cause of long-term disability (R. M. Adibhatla et al., 2008), and more than 30 % of stroke survivors will have severe disability (A. R. Green, 2008). Stroke is primarily a vascular disease with a neurological outcome (A. R. Green, 2008). Stroke can be hemorrhagic caused by rupture of a cerebral blood vessel (12%) or ischemic caused by occlusion of a cerebral artery. Ischemic stroke might be either thrombolic in which a clot or thrombus is formed in a cerebral artery and blocks blood flow at the site of formation or embolic, where a cerebral artery is blocked by a clot formed elsewhere and carried to the brain through the circulation (R. M. Adibhatla et al., 2008).

Cerebral ischemia may be global which results from the transient cessation of blood flow to the brain (as in case of cardiac arrest) and is associated with problems of cognition and memory, sensorimotor deficits, seizures and death (C. K. Petito et al., 1987). Focal cerebral ischemia refers to local interruption of blood flow to the brain due to blockade of a major cerebral artery which results in disruption of glucose and oxygen supply leading to apoptotic and necrotic cell death. In focal ischemia there is an ischemic core surrounded by a “penumbra” region that has partial reduction in blood flow due to collateral arteries. The ischemic core is generally considered unsalvageable, whereas the penumbra may be rescued otherwise the infarct can propagate into the penumbra (R. M. Adibhatla et al., 2008).

On average, the impact of stroke on the nervous tissue is severe as patients with acute ischemic stroke are estimated to lose 120 million neurons, 830 billion synapses and 714 km of myelinated fibers each hour which when compared with the normal rate of neuronal loss during aging would mean that the ischemic brain will age 3.6 years for every hour the stroke is untreated (J. L. Saver, 2006). Despite this, the only treatment of ischemic stroke available is recanalization of the occluded vessel by thrombolysis. The thrombolytic compound tissue plasminogen activator (t-PA), which degrades the fibrin clot blocking the blood flow to the brain tissue, is the

only Food and Drug Administration (FDA)-approved agent for stroke therapy in humans (J. T. Lang and L. D. McCullough, 2008). However, thrombolysis is safe and effective only within 3 hours of the onset of symptoms and is not suitable for many patients (J. F. Maestre-Moreno et al., 2005; E. Juttler et al., 2006). This short time window results in a low treatment rate and warrants safer treatment regimes.

2.2. Mechanism of damage associated with stroke

Although different mechanisms are involved in the pathogenesis of stroke, there is increasing evidence that inflammation accounts for its progression at least acutely (F. C. Barone and G. Z. Feuerstein, 1999; Y. Samson et al., 2005; A. Chamorro and J. Hallenbeck, 2006). During ischemia, the cessation of blood flow, the energy loss and the necrotic cell death initiate an immune response, activate inflammatory cells (microglia/macrophages) and generate reactive oxygen species (ROS) including hydroxyl radical, superoxide anion radical, and hydrogen peroxide. ROS can further stimulate the ischemic cells to release cytokines causing up-regulation of adhesion molecules and mobilization and activation of leukocytes (M. L. Alexandrova and P. G. Bochev, 2005) (Figure 1.1). Also the influx of calcium following ischemia induces the production of ROS (D. Tassoni et al., 2008).

In addition, reperfusion of the occluded vessel leads to the formation of ROS, which stimulate ischemic cells to further secrete cytokines, chemokines, matrix metalloproteinases (MMPs), nitric oxide (NO) and more ROS causing further cell damage and disruption of the blood brain barrier (BBB) and extracellular matrix (H. C. Emsley and P. J. Tyrrell, 2002; G. H. Danton and W. D. Dietrich, 2003). Calcium influx associated with ischemia stimulates phospholipases, which act on membrane phospholipids releasing huge amounts of arachidonic acid (AA). AA is then metabolized by the cyclooxygenase (COX) to produce eicosanoids, which are actively involved in the neuroinflammatory response occurring after ischemia (S. D. Hurley et al., 2002; Y. Gilgun-Sherki et al., 2006).

Focal ischemia is accompanied by an increase in the levels of several cytokines such as tumor necrosis factor (TNF), interleukines IL-1β and IL-6, and several

chemokines such as monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1 alpha (MIP1α)(T. Liu et al., 1994; X. Wang et al., 1994; J. S. Kim et al., 1995; X. Wang et al., 1995; M. A. Soriano et al., 2000; A. Lu et al., 2003; R. Kapadia et al., 2006). All of these inflammatory mediators are known to increase the expression of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), P-selectin and E-selectin on endothelial cells and white blood cells (M. L. Dustin et al., 1986; M. P. Bevilacqua et al., 1987; O. Abbassi et al., 1993; M. J. Eppihimer et al., 1996; G. Stoll et al., 1998)(Figure 1.1).

2.2.1. Reactive oxygen species in ischemia

ROS cause oxidative damage to nucleic acids, proteins, carbohydrates and lipids, normally there are intracellular defense mechanisms to detoxify ROS but when the production of ROS exceeds the cells capacity to detoxify them, there is a decline in physiological functions and progressive cell damage (oxidative stress). The brain is highly sensitive to that condition (R. M. Adibhatla et al., 2008).

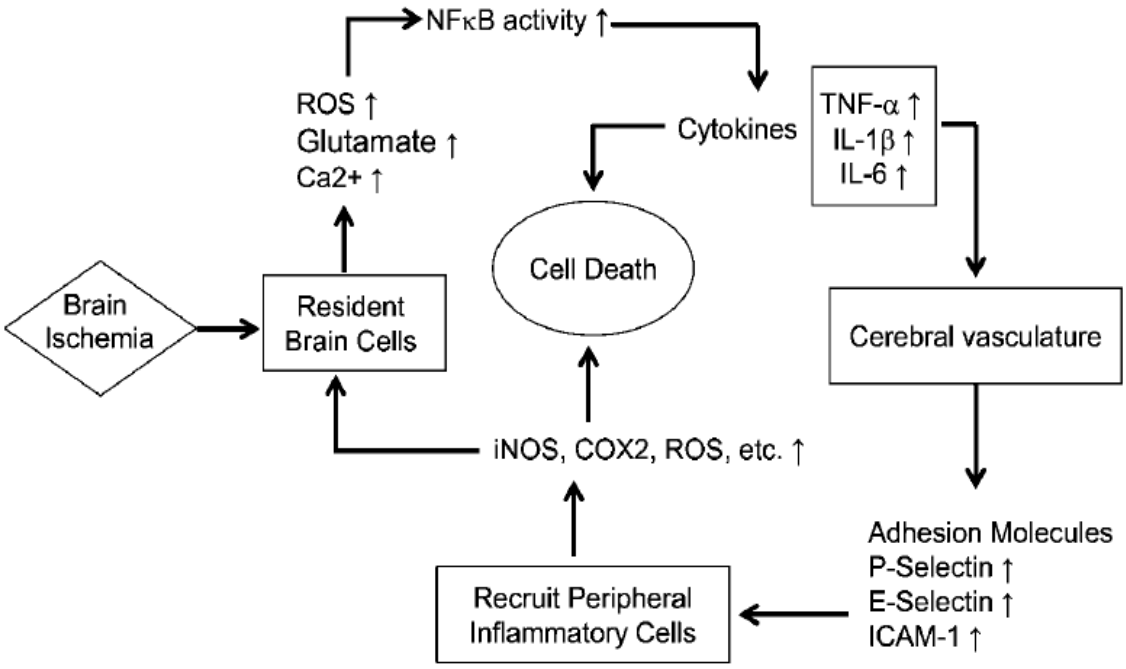


Figure 1.1. Cellular and molecular events following ischemia, after Z. Zheng and M. A Yenari, 2004.

2.2.2. Adhesion molecules in ischemia

Expression of adhesion molecules such as ICAM-1 on endothelial cells and white blood cells is a key step in brain inflammation following stroke that facilitates the adhesion and transendothelial migration of neutrophils, lymphocytes (S. L. Stevens et al., 2002; G. Z. Li et al., 2005) and macrophages into the brain. Leukocytes release several pro-inflammatory cytokines, chemokines and oxygen/nitrogen free radicals, leading to secondary tissue damage within the penumbra (Q. Wang et al., 2007). According to recent evidence, preventing the infiltration of neutrophils or lymphocytes into the ischemic brain ameliorated the ischemic injury (K. Becker et al., 2001; A. Garau et al., 2005). Infiltration of leukocytes into the brain involves rolling, adhesion and transendothelial migration (Q. Wang et al., 2007). Three groups of cell adhesion molecules mediate the interaction between leukocytes and the endothelium: selectins (P-, E- and L-), members of the immunoglobulin superfamily (intracellular adhesion molecule, ICAM-1, vascular cell adhesion molecule, VCAM-1) and integrins (H. C. Emsley and P. J. Tyrrell, 2002).

Blocking selectins (P- and E- selectin) improved stroke outcome (J. Mocco et al., 2002), while, blocking L-selectin did not (M. A. Yenari et al., 2001). Similarly, blocking ICAM-1 with antibodies (Y. Kanemoto et al., 2002), with antisense oligonucleotides (R. Vemuganti et al., 2004) or even with nitric oxide donors preventing elevation of ICAM after ischemia/reperfusion (M. Khan et al., 2006) improved the outcome in experimental stroke. Inhibition of the ischemia-induced upregulation of VCAM was also reported to reduce infarct size (L. H. Zhang and E. Q. Wei, 2003; A. Cervera et al., 2004). Similarly, blockade of neutrophil CD11b after hypoxia was shown to be protective (H. Chen et al., 1994; D. Harmon et al., 2004).

2.2.3. Matrix metalloproteinases in ischemia

In addition to the locally secreted pro-inflammatory cytokines, which act directly on endothelial cells causing increased BBB permeability (G. Y. Yang et al., 1999), there is an increase in the expression and activation of MMPs, plasminogen activators and serine proteases (G. del Zoppo et al., 2000), which are also associated with BBB damage and neuronal injury following experimental ischemia

(J. H. Heo et al., 1999; G. del Zoppo et al., 2000). In addition, inhibition of MMPs reduces infarct size after ischemia (T. Pfefferkorn and G. A. Rosenberg, 2003). However, MMPs may participate in plasticity and recovery at late phases of ischemia probably through an effect on angiogenesis and neurovascular remodeling (B. Q. Zhao et al., 2006).

2.2.4. Transcription factors in ischemia

Transcription factors play an important role in controlling gene expression under normal and inflammatory conditions. Several transcription factors were reported to be induced following ischemia, some of which are known to prevent ischemic neuronal damage and/or promote ischemic tolerance such as hypoxia inducible factor-1 (HIF-1), c-fos and peroxisome proliferator-activated receptor (PPAR-γ) (K. Tanaka et al., 2000a; K. Tanaka et al., 2000b; S. Cho et al., 2001; K. Maeda et al., 2001). In contrast, the induction of several other transcription factors promotes inflammation and neuronal cell death following ischemia. Examples of the latter group are activating transcription factor-2 (ATF-2), signal transducer and activator of transcription 3 (STAT3), nuclear factor kappa B (NF-κB), early growth response-1 (Egr1) and CCAAT/enhancer binding protein (C/EBP) beta (C/EBPβ) (A. M. Planas et al., 1996; M. Bergeron et al., 1999; C. Iadecola et al., 1999; B. R. Hu et al., 2000; I. M. Johansson et al., 2000; D. Stephenson et al., 2000; S. F. Yan et al., 2000; K. Tanaka et al., 2000a; K. Tanaka et al., 2000b; A. J. Williams et al., 2003; A. Nurmi et al., 2004; R. Kapadia et al., 2006; K. Tureyen et al., 2007).

1.2.4.a. Nuclear factor kappa B (NF-κB) in ischemia

Nuclear factor (kappa) B (NF-κB) was first described in 1986 as a nuclear factor necessary for immunoglobulin kappa light chain transcription in B cells (Z. Zheng and M. A. Yenari, 2004). It represents a family of proteins that share a highly conserved 300 amino acid N-terminal domain called Rel homology domain (RHD). The RHD contains a nuclear localization sequence (NLS) and is involved in dimerization, sequence specific DNA binding and interaction with the inhibitory IκB proteins (S. Ghosh et al., 1998). NF-κB/Rel proteins include five members, which

can form homodimers or heterodimers: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel (G. Bonizzi and M. Karin, 2004).

In most cell types, NF-κB dimers are retained in the cytoplasm (in an inactive state) by IκBs, which are specific inhibitors that bind to the RHD and interfere with its NLS function. The translocation of NF-κB dimers from the cytoplasm to the nucleus occurs via the classical pathway and an alternative pathway (G. Bonizzi and M. Karin, 2004; M. Karin and F. R. Greten, 2005).

In the classical pathway, pro-inflammatory cytokines activate the IκB kinase (IKK) complex which consists of the IKK1 and IKK2 catalytic subunits and the IKK3 regulatory subunit (NEMO for 'NF-κB essential modulator'). In this pathway, the activated IKK complex, predominantly acting through IKK2 in an IKK3-dependent manner, catalyzes the phosphorylation of IκB followed by polyubiquitination and subsequent proteasomal degradation of IκBs releasing NF-κB dimers (p50–RelA dimer) which then translocate to the nucleus. Nuclear NF-κB then binds DNA and activates gene transcription (S. Ghosh and M. Karin, 2002).

In the alternative pathway of NF-κB activation, IKK1 homodimers phosphorylate NF-κB2/p100 dimers followed by polyubiquitination and proteasomal degradation (G. Xiao et al., 2001) (Figure 1.2).

NF-κB controls the transcription of several pro-inflammatory cytokines such as IL-1β, TNF and granulocyte-macrophage colony stimulating factor (GM-CSF), and chemokines as IL-8, MIP-1α, which attract inflammatory cells to the site of stimulation (P. J. Nelson et al., 1993; N. Mukaida et al., 1994; U. Siebenlist et al., 1994; A. Ueda et al., 1994). NF-κB also induces the expression of the adhesion molecules ICAM-1, VCAM-1 and E-selectin on endothelial cells, which facilitate the adherence of inflammatory cells (A. van de Stolpe et al., 1994; M. F. Iademarco et al., 1995). Thus, NF-κB proteins lie in the heart of most inflammatory responses.

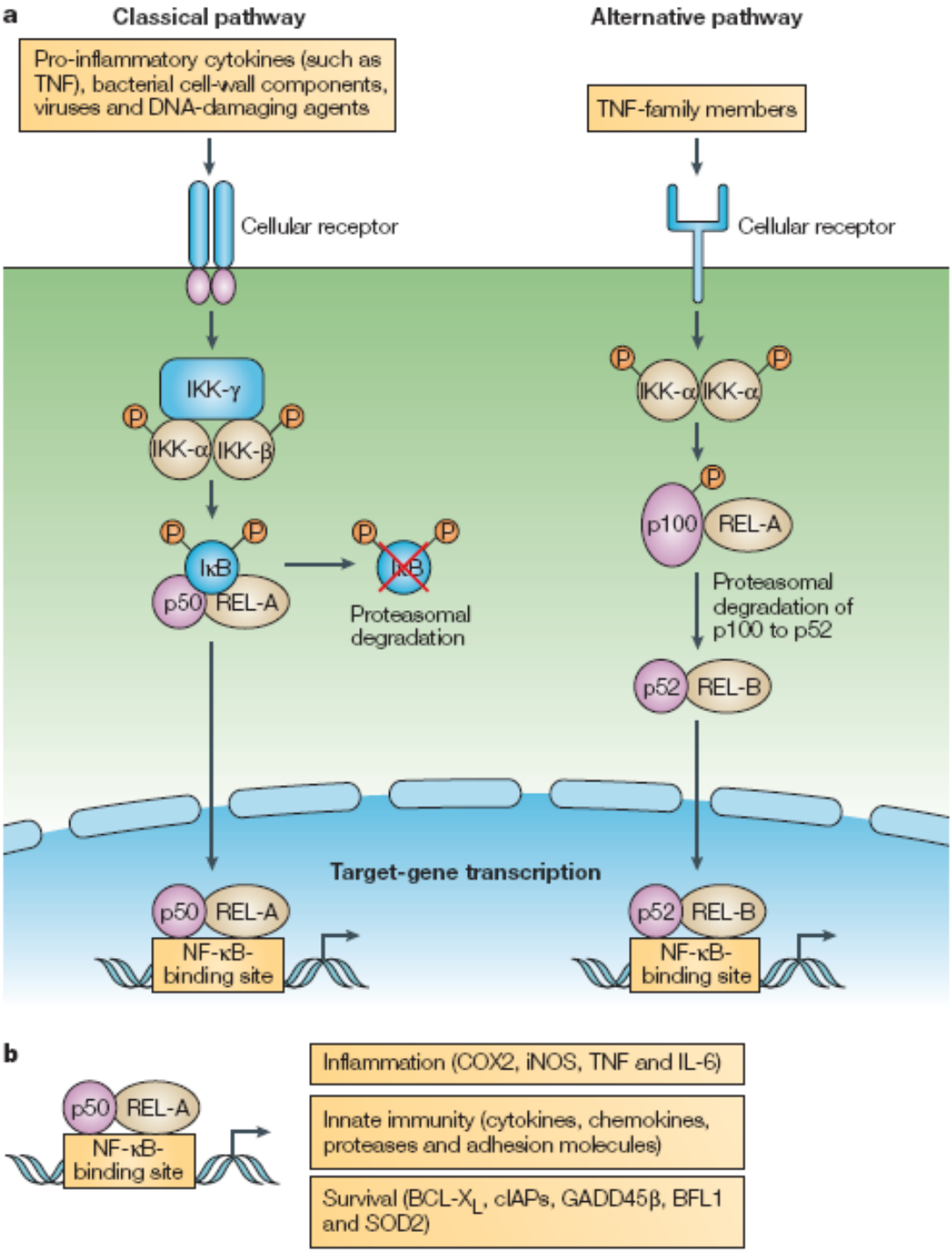


Figure 1.2. NF-κB activation, after M. Karin and F. R. Greten 2005.

Some of the down-stream target genes of NF-κB, e.g., IL-1β and TNF, can re-activate NF-κB itself resulting in a positive feedback activation loop of NF-κB (P. J. Barnes and I. M. Adcock, 1998). However, the transcription of the IκB gene is regulated by NF-κB and activation of NF-κB induces the expression of IκB, which enters the nucleus, binds NF-κB and transports it out to the cytoplasm forming a negative feedback control of the activity of NF-κB (Z. Zheng and M. A. Yenari, 2004).

Activation of NF-κB was reported following ischemia (A. Schneider et al., 1999; H. Seegers et al., 2000; D. Stephenson et al., 2000). Inhibition of NF-κB activity reduces the infarct size (A. Nurmi et al., 2004) and mice deficient in p50 develop smaller infarcts after focal ischemia (A. Schneider et al., 1999). The activation of NF-κB following ischemia might involve TNF (G. I. Botchkina et al., 1999), IL-1 (S. L. Dunn et al., 2002) and may depend on ionotropic glutamate receptors and L-type voltage-gated calcium channels because antagonists of these channels were reported to inhibit activation and translocation of NF-κB (W. Shen et al., 2002).

1.2.4.b. Other transcription factors in ischemia

Activator protein-1 (AP-1) is a heterodimer comprised of activating transcription factor-2 (ATF-2), c-fos or c-jun which bind to a specific DNA sequence that regulates the expression of several genes known as late response genes (Q. Wang et al., 2007). AP-1 can be activated by TNF and IL-1β and following cerebral ischemia (V. L. Woodburn et al., 1993; M. Dragunow et al., 1994). HIF-1 is known to be upregulated after stroke leading to activation of iNOS and promoting neuronal cell death (C. Iadecola et al., 1995). In addition, C/EBPβ was shown to be upregulated following ischemia (V. L. Raghavendra Rao et al., 2002) and is known to control the expression of IL-6, IL-1β, IL-8, IL-12, TNF and MCP-1 (M. N. Bradley et al., 2003).

Ischemia induces cerebral Egr1 expression (J. H. Yi et al., 2007), which regulates the expression of several inflammatory genes (S. F. Yan et al., 2000). PPARγ is known to influence immune and inflammatory functions in macrophages, T cells, B

cells, dendritic cells and endothelial cells (P. Gosset et al., 2001; D. C. Jones et al., 2002; C. K. Glass and S. Ogawa, 2006). Agonists of PPARγ exhibit anti-inflammatory properties after ischemia (P. R. Devchand et al., 1996; K. Setoguchi et al., 2001).

2.2.5. Role of cytokines in ischemia

Cytokines act as mediators for regulating the innate and adaptive immune systems (R. M. Adibhatla et al., 2008). They are pleiotropic (acting on different cell types), multifunctional (the same cytokine can regulate different functions) and redundant (different cytokines can carry out the same function because they use shared intracellular signaling pathways) (J. Huang et al., 2006). Some cytokines are pro-inflammatory (e.g., TNF, IL-1, interferon-γ IFN-γ, IL-12, IL-18 and granulocyte-macrophage colony stimulating factor GM-CSF), while others are anti-inflammatory (e.g., IL-4, IL-10, IL-13, IFN-α and transforming growth factor-ß TGF-ß) (R. M. Adibhatla et al., 2008).

Cytokine release in the brain is increased in response to ischemia and other injuries. The inflammatory response often includes glial activation, which suppresses the injury process. However, an unchecked inflammatory response may increase the injury process (Q. Wang et al., 2007).

Stroke is followed by upregulation of several cytokines in the brain produced by immune cells and neurons and glia (T. Liu et al., 1994; T. Sairanen et al., 2001), some of these cytokines such as IL-1β (A. S. Haqqani et al., 2005) and TNF (T. Liu et al., 1994) exacerbate the injury while others such as IL-6, IL-10 and TGF-β may be neuroprotective (S. M. Allan and N. J. Rothwell, 2001).

1.2.5.a. Role of TNF in ischemia

Following ischemia, there is an increase in TNF as well as its two receptors p55 and p75 in the brain. p55 contains a death domain and mediates most of the effects of TNF including inflammatory response, cytotoxicity and AA release (C. X. Wang and A. Shuaib, 2002). p75 may mediate a protective effect after ischemia (Y. Shen et

al., 1997). Antagonizing TNF by a neutralizing antibody showed beneficial effects after ischemia (S. D. Lavine et al., 1998) and mice deficient in TNF had smaller infarcts (A. Martin-Villalba et al., 2001). In addition, infusion with TNF had increased infarct volumes following focal cerebral ischemia (F. C. Barone et al., 1997).

On the other hand, mice deficient in p55 and p75 had larger infarcts (A. J. Bruce et al., 1996) which would raise the possibility of the existence of another unidentified TNF receptor (N. J. Rothwell and G. N. Luheshi, 1996). Pretreatment of hippocampal cultures with TNF protected against OGD induced neuronal damage while its application after OGD increased the damage (G. J. Wilde et al., 2000). This finding resembles ischemic preconditioning where a short sub-lethal ischemic insults provide protection against subsequent severe ischemic injury (R. M. Adibhatla et al., 2008). The effect of TNF might be mediated by the production of ROS, activation of NF-κB and up-regulation of manganese superoxide dismutase (Mn-SOD), which may provide defense against subsequent ROS generation. However, addition of TNF after injury would stimulate ROS generation and aggravate the damage (G. J. Wilde et al., 2000).

1.2.5.b. Role of interleukin-1 (IL-1) family in ischemia

IL-1 proteins include IL-1α, IL-1β (both of which interact with IL-1 receptor type I mediating all IL-1 signaling or IL-1 receptor type II which is believed to be a non-signaling or decoy receptor). The third member of the IL-1 family is the endogenous receptor antagonist (IL-1ra) that binds to and blocks IL-1 receptor type I and antagonizes the effects of IL-1α and β (N. J. Rothwell, 1999). Interestingly, treatment with IL-1ra was protective against ischemia (H. C. Emsley et al., 2005), while its knockout animals suffered from larger infarcts (E. Pinteaux et al., 2006).

The brain expresses mainly IL-1β which is up-regulated after stroke, it was shown that administration of IL-1β increased brain damage in ischemic rats (Y. Yamasaki et al., 1995). More interestingly, individual knockouts in IL-1α or IL-1β had similar infarcts as wild-types after MCAO while double knockouts had smaller infarcts

indicating a compensatory response between IL-1α and IL-1β (H. Boutin et al., 2001).

1.2.5.c. Role of other cytokines in ischemia

Ischemia is followed by an up-regulation of IL-6 (X. Wang et al., 1994). IL-6 has an anti-inflammatory effect by inhibiting the synthesis of TNF and IL-1, by inducing the synthesis of TNF binding protein (which binds TNF and prevent it from interacting with its receptors) and also by inducing the synthesis of IL-1ra (B. E. Barton, 1997).

IL-10 has anti-inflammatory effects by blocking the production of IL-1 and TNF (Q. Wang et al., 2007) and is upregulated in experimental stroke (K. Strle et al., 2001). In addition, exogenous administration of IL-10 in ischemia had beneficial effects (P. A. Spera et al., 1998). TGF-β1 contributes to the recovery of ischemic stroke and was shown to protect mouse brains from ischemic stroke (L. Pang et al., 2001).

Expression of chemokines such as MCP-1 and macrophage inflammatory protein-1α (Y. Chen et al., 2003) after ischemia has a deleterious role by increasing leukocyte infiltration (H. C. Emsley and P. J. Tyrrell, 2002).

2.2.6. Role of nitric oxide in ischemia

Nitric oxide (NO) is involved in many physiological processes such as neuronal communication, host defense and regulation of vascular tone. It can be produced by three different synthases: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). iNOS is expressed mainly by cells involved in inflammatory responses such as leukocytes, microglia and astrocytes (Q. Wang et al., 2007). iNOS expression and NO production were induced after ischemia (C. Iadecola et al., 1995).

2.2.7. Role of arachidonic acid and its metabolites in ischemia

In the brain, energy is supplied by metabolism of glucose and oxygen for the phosphorylation of ADP to ATP, which is used to maintain the intracellular homeostasis and transmembrane ion gradients of sodium, potassium, and calcium. However, energy failure following stroke leads to rapid loss of ATP and uncontrolled leakage of ions across the cell membrane resulting in an increase in intracellular

calcium and membrane depolarization and release of neurotransmitters such as glutamate and dopamine (R. M. Adibhatla and J. F. Hatcher, 2005). The released glutamate and the increase in intracellular calcium during cerebral ischemia activate phospholipases releasing free fatty acid (FFA) such as AA (H. Katsuki and S. Okuda, 1995). AA can stimulate the expression of TNF and IL-1ß (M. Hughes-Fulford et al., 2006). Both TNF and IL-1ß can activate phospholipases suggesting a self-re-enforcing cycle (R. M. Adibhatla et al., 2008).

The brain content of lipids (36 - 60 %) is the second highest in the body next to adipocytes and comprises polyunsaturated fatty acids (mainly AA or docosahexaenoic acid DHA) (D. Tassoni et al., 2008). AA is released by cytosolic phospholipase A2 (cPLA2) from membrane phospholipids (A. A. Farooqui and L. A. Horrocks, 2006). DHA is liberated by the action of plasmalogen-selective phospholipase A2 (PlsEtn-PLA2) (Y. Hirashima et al., 1992). The liberated AA and DHA can be either reincorporated in neural membrane phospholipids by reacylation reactions or oxidized by several enzymatic and nonenzymatic mechanisms (S. I. Rapoport, 1999).

The enzymatic peroxidation of AA produces eicosanoids, which play important roles, in regulating signal transduction, gene transcription processes, and in inducing and maintaining the acute inflammatory responses (J. W. Phillis et al., 2006) (Figure 1.3). Eicosanoids include prostaglandins produced by cyclooxygenases (COX), thromboxanes and leukotrienes produced by lipoxygenases (LOX), and epoxyeicosatrienoic produced by epoxygenases (EPOX). Furthermore, COX and LOX metabolize DHA to docosanoids which antagonize the effects of eicosanoids and modulate leukocyte trafficking and downregulate the expression of cytokines (S. Hong et al., 2003). Docosanoids include resolvins, docosatrienes and neuroprotectins (J. W. Phillis et al., 2006). The reactions catalyzed by COX, LOX and EPOX also produce ROS.

Nonenzymatic peroxidation of AA and DHA produces 4-hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE), respectively, which are important mediators of neural cell damage (H. Esterbauer et al., 1991). In addition, free radical mediated

nonenzymatic oxidation of AA and DHA produces isoprostanes (L. J. Roberts, 2nd et al., 2005) and neuroprostanes (N. G. Bazan, 2005), respectively, which are both implicated in oxidative stress (Figure 1.3).

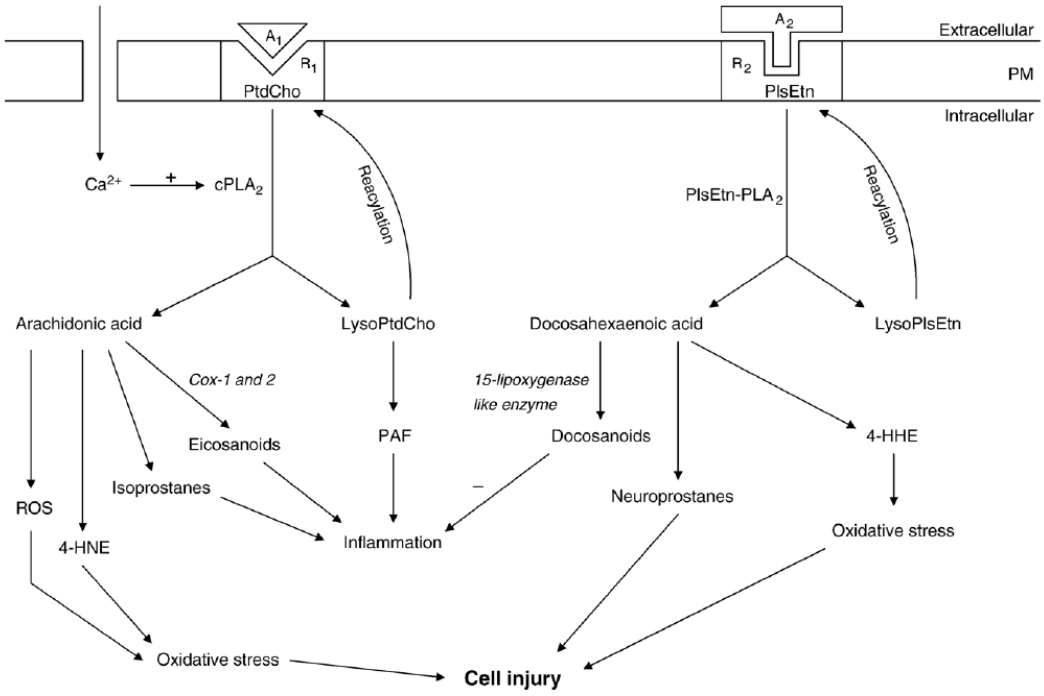


Figure 1.3. Release of arachidonic acid and docosahexaenoic acid and their metabolism, after J. W. Phillis et al., 2006.

Agonist (A1 and A2); receptors (R1 and R2); plasma membrane (PM); phosphatidylcholine (PtdCho); ethanolamine plasmalogen (PlsEtn); lysophosphatidylcholine (LysoPtdCho); ethanolamine lysoplasmalogen (LysoPlsEtn); platelet-activating factor (PAF); calcium (Ca^{2+}); cytosolic phospholipase A2 (cPLA2); plasmalogen-selective phospholipase A2 (PlsEtn-PLA2); cyclooxygenase-1 and 2 (COX-1 and 2); 4-hydroxynonenal (4-HNE); and reactive oxygen species (ROS).

1.2.7.a. Role of phospholipase A-2 (PLA-2) in ischemia

Phospholipase A-2 (PLA-2) isozymes include the calcium independent iPLA-2, the secretory sPLA-2 and the calcium-dependent cytosolic cPLA-2. cPLA-2 preferentially cleaves phospholipids containing AA while iPLA-2 and sPLA-2 have no specificity (R. M. Adibhatla et al., 2003; S. Akiba and T. Sato, 2004).

Calcium influx associated with ischemia/reperfusion stimulates PLA2 activity leading to an increase in the free AA (D. Tassoni et al., 2008). cPLA-2 was up-regulated after stroke (D. T. Stephenson et al., 1994), while cPLA-2 knockout mice had smaller infarcts (J. V. Bonventre et al., 1997).

cPLA-2 can be induced by several pro-inflammatory cytokines: *in vitro* exposure of astrocyte cultures to IL-1β induced the expression of cPLA-2 (N. Stella et al., 1997), while exposure of an astrocytoma cell line to TNF increased the phosphorylation of cPLA-2 through activation of the MAP kinase pathway (M. Hernandez et al., 1999).

1.2.7.b. Role of cyclooxygenase (COX) in ischemia

Cyclooxygenases are correctly called prostaglandin-endoperoxide synthase, because they catalyzes the conversion of AA into prostaglandin G_2 (PGG_2), and then to prostaglandin H_2 (PGH_2), which is the precursor of several other prostanoids, including prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), and thromboxanes (E. Candelario-Jalil and B. L. Fiebich, 2008).

COX exists in the isoforms COX-1 and COX-2. COX-3 is a splice variant of COX-1 (B. Kis et al., 2006). COX-1 and COX-2 share about 60 % amino acid identity but differ in their expression pattern (E. Candelario-Jalil and B. L. Fiebich, 2008). COX-1 is constitutively expressed in many cells (J. M. Schwab et al., 2002). It is thought to mediate physiological responses (T. Takemiya et al., 2007) and to have a protective role since mice deficient in COX-1 showed increased sensitivity to ischemia. This protective effect is probably caused by regulating cerebral blood flow (C. Iadecola et al., 2001).

In contrast, COX-2 is an inducible isoform, which can be upregulated in response to acute seizures and ischemia (T. Takemiya et al., 2007) and in response to inflammatory cytokines, bacterial lipopolysaccharide (LPS) and tumor promoters (E. Candelario-Jalil and B. L. Fiebich, 2008). COX-2 mediates its toxic effect through PGE_2 (Y. Manabe et al., 2004) and the production of free radicals (E. Candelario-Jalil and B. L. Fiebich, 2008). COX-2 expression is upregulated after experimental ischemia (S. Nogawa et al., 1997) and this was associated with increased PGE_2

levels and neuronal apoptosis (R. C. Li et al., 2003). Indeed, COX-2 deficient mice suffered less brain injury following MCAO (C. Iadecola et al., 2001) and treatment with COX-2 inhibitors decreased hippocampal neuronal damage after ischemia (T. Sasaki et al., 2004).

1.2.7.c. Role of prostaglandin E synthase (PGES) in ischemia

In the biosynthetic pathway leading to PGE_2 formation, phospholipase A2 (cytosolic or secretory) acts on membrane phospholipids releasing AA which is converted to PGH_2 by COX. PGH_2 is then isomerized to PGE_2 by prostaglandin E synthase (A. C. de Oliveira et al., 2008) (Figure 1.4). Prostaglandin E synthases may be microsomal PGESs (mPGES-1 and mPGES-2) or cytosolic (cPGES)(N. Tanikawa et al., 2002). Microsomal prostaglandin E synthase-1 (mPGES-1) is functionally coupled with COX-2 (M. Murakami et al., 2000).

Expression of mPGES-1 is of major importance in pathophysiological events in which COX-2-derived PGE_2 plays a crucial role because it has the most terminal position in the PGE_2-synthesing pathway, the highest magnitude of upregulation among other PGE_2-synthesizing enzymes and a long duration of mRNA expression (A. C. de Oliveira et al., 2008). mPGES-1 expression was increased in neurons, microglia and endothelial cells after ischemia (Y. Ikeda-Matsuo et al., 2006). Moreover, mPGES-1 deficient mice had smaller infarcts, less edema and less apoptosis following ischemia (Y. Ikeda-Matsuo et al., 2006).

1.2.7.d. Role of prostaglandin E_2 (PGE_2) in ischemia

PGE_2 is responsible for the neurotoxic effects of COX-2 (Y. Manabe et al., 2004). PGE_2 acts on four G-protein coupled receptors (EP1-4), EP2, EP3 and EP4 being neuroprotective (M. Bilak et al., 2004; L. McCullough et al., 2004; A. S. Ahmad et al., 2005). EP1 mediates the neurotoxic effect of PGE_2 (T. Kawano et al., 2006) (Figure 1.4). COX-2 inhibitors decreased hippocampal neuronal damage after ischemia by reducing PGE_2 concentration (M. Nakayama et al., 1998), indicating that PGE_2 produced by COX-2 may be a stimulator of neuronal damage. PGE_2 aggravated neuronal damage following ischemia (J. Thornhill and M. Smith, 1998)

and the level of PGE_2 was increased after ischemia (E. Candelario-Jalil et al., 2003; C. Yokota et al., 2004).

Brain PGE_2 is synthesized and derived from neurons (P. Ciceri et al., 2002), microglia (N. P. Turrin and S. Rivest, 2004), astrocytes (M. Zonta et al., 2003) or endothelial cells (T. Takemiya et al., 2007). However, Takemiya et al., proposed that PGE_2 released from endothelial cells may promote calcium-dependent glutamate release from astrocytes leading to an increase in neuronal calcium level and neuronal death (T. Takemiya et al., 2007). Others consider PGE_2 released by activated microglia to be of major importance for the initiation, propagation and modulation of brain inflammation (B. Liu and J. S. Hong, 2003). In contrast, PGE_2 administration protected against neuronal death induced by LPS through reduction of NO release from microglia and ROS from neurons (E. J. Kim et al., 2002).

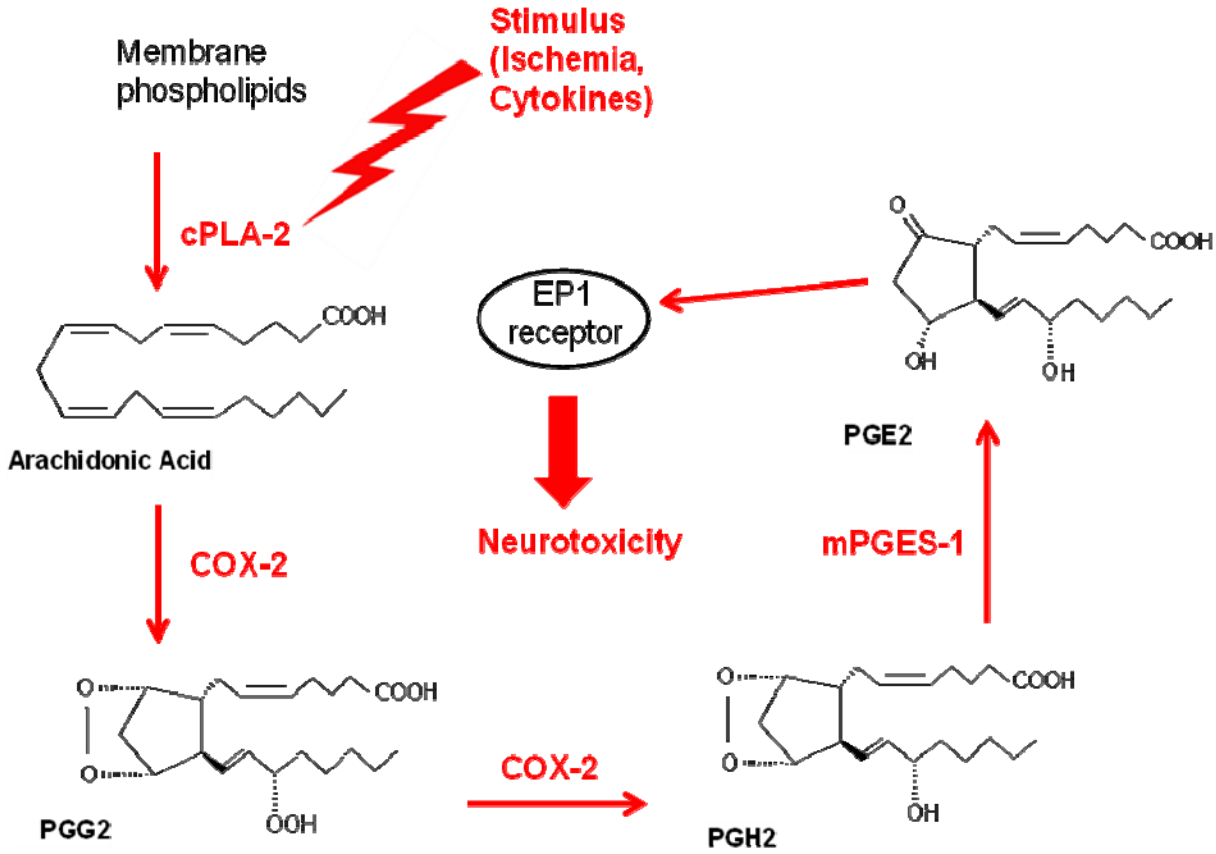


Figure 1.4. Activation of arachidonic acid cascade in ischemia

2.2.8. Role of High Mobility Group Box-1 in ischemia

HMGB1 is a 30 kD, abundant, non-histone nuclear protein which derives its name from its characteristic fast migration in electrophoresis (M. Bustin, 2001), HMGB1 is highly expressed in all eukaryotic cells and is conserved through evolution (99% identity in mammals) (S. Muller et al., 2004). Structurally, its 215 residues are organized into two DNA-binding domains (Box A and B) each containing about 80 amino acids in an L-shaped fold and a negatively charged C-terminus (H. M. Weir et al., 1993; I. E. Dumitriu et al., 2005) (Figure 1.5).

In its resting state, the long acidic tail of HMGB1 interacts with basic stretches in the box A and box B shielding them from other interactions that might occur before HMGB1 binds DNA (S. Knapp et al., 2004). Box B is responsible for many of the pro-inflammatory effects of HMGB1 including cytokine release (J. Li et al., 2003) while box A acts as a specific antagonist for the interaction between HMGB1 and RAGE (M. E. Bianchi and A. A. Manfredi, 2007).

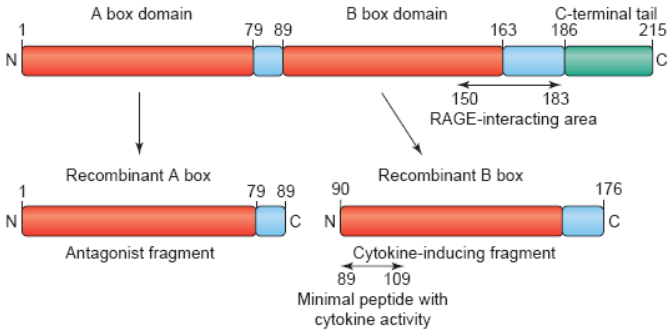


Figure 1.5. Structure of HMGB1, after I. E. Dumitriu et al., 2005.

Most cells contain about a million molecules of HMGB1 which bind DNA without sequence specificity and induce bends in DNA enabling the physical interaction between DNA and various factors such as NF-κB and hormone receptors (M. E. Bianchi and A. Manfredi, 2004), thus regulating the transcription of many genes (J. S. Park et al., 2003). HMGB1 is an extremely mobile protein resting on a specific DNA site only for fractions of a second (R. D. Phair et al., 2004). In addition, some

cells express HMGB1 in the plasma membrane (in addition to the nucleus). HMGB1 is referred to as amphoterin which mediates neurite outgrowth (J. Parkkinen et al., 1993) and tumor cell metastasis (A. Taguchi et al., 2000).

HMGB1 can be released passively from necrotic cells (P. Scaffidi et al., 2002) or actively from immune cells (H. Yang et al., 2004) (Figure 1.6). Passively released HMGB1 from necrotic (but not from apoptotic) cells serves as a danger signal that alerts the immune system to the presence of injury (K. L. Rock et al., 2005). Passive release of HMGB1 may depend on the activation of poly(ADP)-ribose polymerase enzyme (PARP) after DNA damage which promotes the translocation of HMGB1 from the nucleus to the cytosol (D. Ditsworth et al., 2007).

HMGB1 can be also actively secreted from monocytes or macrophages in response to LPS, TNF, IL-1β and interferon-γ (H. Wang et al., 1999) as a delayed mediator of inflammation (H. Wang et al., 1999). In living cells HMGB1 traffics between the nucleus and cytosol (T. Bonaldi et al., 2003). However, upon stimulation, lysine residues in HMGB1 are acetylated and this blocks its import into the nucleus. Acetylated HMGB1 is then packaged into secretory lysosomes before being released extracellularly (S. Gardella et al., 2002). Extracellular HMGB1 activates monocytes to release more cytokines thus prolonging inflammation (U. Andersson et al., 2000).

Thus, HMGB1 is now considered a cytokine because it is secreted by activated immune cells, mediates systemic inflammatory responses and activates prototypical inflammatory responses in immune cells and endothelial cells (M. T. Lotze and K. J. Tracey, 2005). In addition, HMGB1 was reported to be involved in ischemic injury in several animal models (R. S. Goldstein et al., 2006; J. B. Kim et al., 2008; J. Qiu et al., 2008) and in humans (R. S. Goldstein et al., 2006).

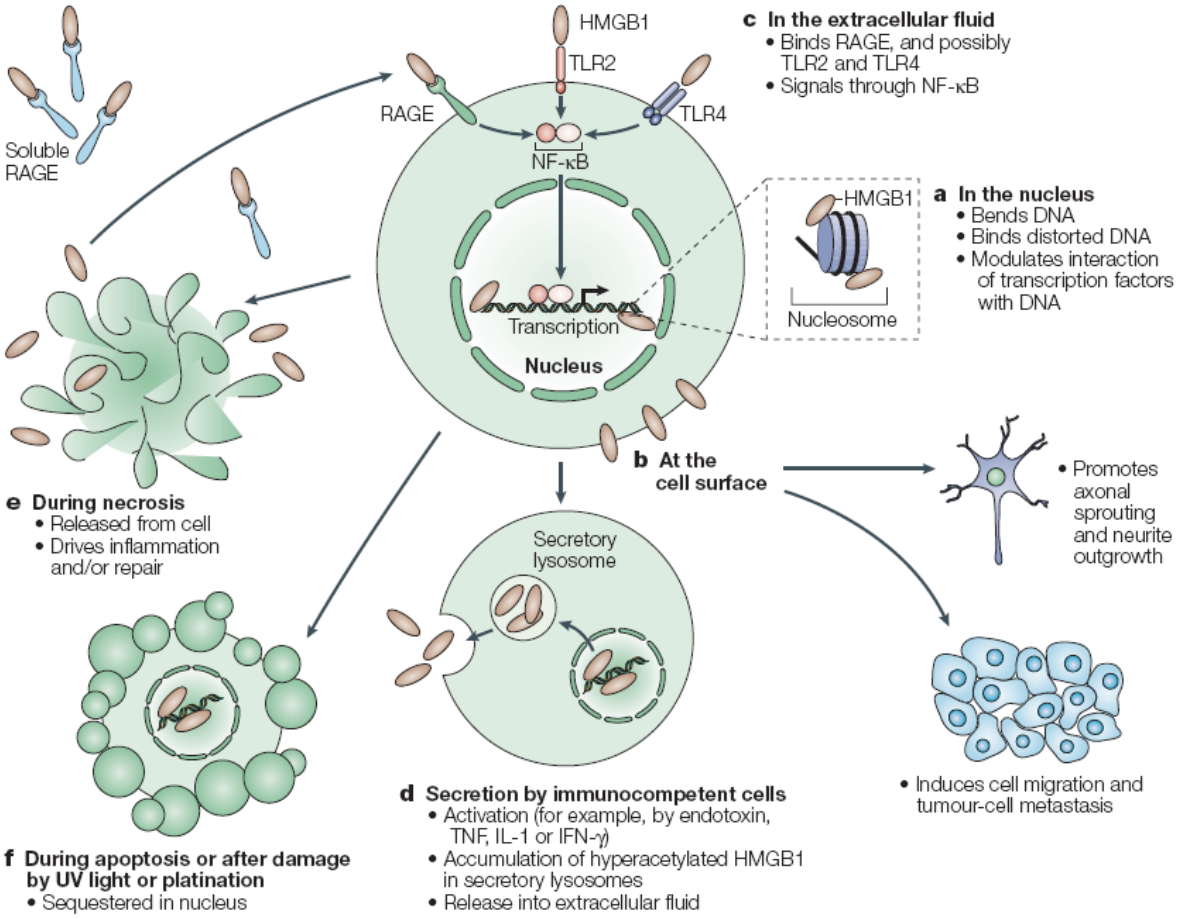


Figure 1.6. Release of HMGB1, after M. T. Lotze and K. J. Tracey, 2005.

HMGB1 may signal through TLR-2, TLR-4 and RAGE. However signaling through RAGE and the TLRs is fast, while HMGB1 induces a delayed response in macrophages which suggest the involvement of another yet to be discovered receptor (M. T. Lotze and K. J. Tracey, 2005).

1.2.8.a. Role of Receptor for Advanced Glycation Endproducts in ischemia

RAGE, the first receptor identified for HMGB1 (M. T. Lotze and K. J. Tracey, 2005), is a member of the immunoglobulin superfamily of cell surface receptors (A. Bierhaus et al., 2005) that is activated by several ligands including HMGB1 but also by advanced glycation end products (AGEs), S100 proteins, and amyloid β-peptide

(Aβ) (M. P. Fink, 2007). RAGE is expressed by monocytes and macrophages, dendritic cells (DCs), endothelial cells and vascular smooth muscle cells (A. M. Schmidt et al., 2001). In the brain, RAGE is present on neurons, glia and endothelial cells (S. D. Yan et al., 1996; R. Deane et al., 2003; O. Arancio et al., 2004; A. Bierhaus et al., 2004). RAGE can be expressed as both a transmembrane receptor and as a soluble molecule (sRAGE) which can block the action of RAGE ligands such as HMGB1 (M. T. Lotze and K. J. Tracey, 2005). RAGE expression was reported to be induced after ischemia and a specific antibody against RAGE reduced the damage caused by OGD (P. Pichiule et al., 2007; D. X. Zhai et al., 2008).

Aim of the study

Ischemia is known to induce the upregulation of several cytokines, transcription factors and AA. AA and its metabolites play an important role in inflammatory processes especially in the brain. Furthermore, the transcription factor NF-κB is essential for the regulation of the transcription of several genes involved in inflammation. In addition, HMGB1, the late mediator of inflammation was reported to have a role in ischemia.

Our present study aims to investigate whether NF-κB regulates the transcription of genes involved in AA metabolism and whether inhibiting NF-κB and AA metabolism could provide a new approach for the treatment of ischemia. We would also explore the role of HMGB1 in mediating the toxic effects observed after ischemia and also study a potential beneficial effect of blocking HMGB1 mediated effects as a therapy of ischemia.

2. Materials and methods

2.1. Cell culture

Materials:

Hank's balanced salt solution (HBSS)	PAA
DNase I	Roche
Bovine serum albumin (BSA)	Roth
Dubellco's modified eagles medium (DMEM)	PAA
Fetal bovine serum (FBS)	PAA
L-glutamine	Gibco
Penicillin/Streptomycin	Gibco
Trypsin-EDTA	Gibco
Phosphate balanced saline (PBS)	Gibco
Glucose	Merck
Hepes	Roth
Neurobasal medium	Invitrogen/Gibco
B27 supplement	Invitrogen/Gibco
Poly-D-lysine	Sigma
Diphtheria toxin	Sigma
Clodronate containing liposomes (25 % W/V)	
PBS containing liposomes	
Sieve (40 µm)	BD Falcon

ACK buffer (ERYLYSE for lysis of erythrocytes and blood cells)

NH_4Cl	2.07g	J.T. Baker
$KHCO_3$	250 mg	Ferak berlin
Na_2EDTA	9.3 mg	Serva
Add 200 ml dH_2O		
sterile filtered		

NS-398	Sigma
SC-51089	Biomol
Lipopolysaccharide (LPS)	Sigma
TNF	Sigma
HMGB1	HMGbiotech

2.1.1. Preparation of glial cultures (Astrocytes/Microglia)

Glia were prepared from NMRI, RAGEko or CD11b-DTR mice at postnatal day P2 or P3 as follows:

Ten to twelve animals were sacrificed by CO_2 inhalation and heads were collected in cold HBSS. Then the brains were extracted under sterile conditions, cut into small pieces with a scalpel and treated with 25 ml warm HBSS containing 0.3 % BSA, 0.004 % DNase and 0.025 % trypsin (sterile filtered) for 15 min at 37 °C with occasional shaking. The reaction was stopped by the addition of 25 ml DMEM full medium (containing 10 % FBS, 0.5 mM L-glutamine and 100 IU/ml penicillin and 100 µg/ml streptomycin). Cells were centrifuged at 1,000 x g for 7 minutes. The cell pellet was resuspended in 15 ml warm HBSS containing 0.3 % BSA, 0.004 % DNase (sterile filtered) and incubated at room temperature for 10 min. After tissue fragments settled down, the supernatant was carefully transferred to another tube and kept at 37 °C while the pellet was resuspended again in the same solution and incubated for another 10 min. The clear supernatant was combined with that from the previous step. This procedure was repeated to get a total of three resuspension steps. The combined supernatant was then centrifuged at 1,000 x g for 7 minutes and the pelleted cells were resuspended in 10 ml warm growth medium. Using a fire-polished Pasteur pipette, cell clusters were separated into a single cell-suspension and the volume adjusted to 15 ml per 2 brains. Cells were plated into a 75 cm^2 flask and incubated at 37 °C under 5 % CO_2. The cell culture medium was changed every 5 days and cells were splitted at 90 % confluency.

For CD11b-DTR mice the cells were prepared from each mouse individually and cultured in 25 cm^2 flasks. Meanwhile, a part of the tail was collected for genotyping.

For splitting of cells, medium was removed and the cells were washed once with warm PBS. Trypsin-EDTA (2 ml per 75 cm^2 flask or 1 ml per 25 cm^2 flask) was added and the flask was incubated at 37 °C for 5-10 min until cells detached. Then the reaction was stopped by adding 8 ml full medium (containing FBS) and the cells were collected in a tube, centrifuged at 1,000 x g for 7 minutes and the cell pellet

was then resuspended in 12 ml full growth medium. Cells were plated (500 µl in each well in a 24-well plate or 2 ml in each well of 6-well plate). Neurons were plated on glia 7 days after splitting.

2.1.2. Depletion of microglia from mixed glial cultures

Mixed glial cultures prepared from CD11b-DTR mice were genotyped and the DTR-positive cells were treated at day *in vitro* (DIV) 5-6 with diphtheria toxin 100 ng/ml or PBS for 24 h in DMEM (without FBS). After 9-10 days, neurons were plated on glial cells and the cultures were stained for Iba-1 to detect the depletion of microglia.

In another set of experiments, mixed glial cultures were prepared from NMRI mice and were treated at DIV 5-6 with clodronate or PBS containing liposomes (which were kindly provided from Dr. N. Van Rooijen, Department of Molecular cell Biology, Vumc, FdG, Amesterdam, The Netherlands) for 24 hours in full DMEM medium (0.2 ml liposome solution in 3 ml DMEM medium). After 9-10 days, neurons were plated on glial cells and the cultures were stained for Iba-1 to detect the depletion of microglia.

2.1.3. Cortical Neuron Preparation

Primary cortical neurons were prepared from embryonic NMRI mice at embryonic day 16 (E16) as follows:

The pregnant mouse was sacrificed by CO_2 inhalation and the embryos were collected into ice cold dissection buffer (0.74 % Hepes and 0.6 % Glucose in PBS, pH 7.3, sterile filtered).

Under sterile conditions, the cerebral cortices were dissected under an operational microscope while meninges, hippocampus and olfactory bulb were removed and discarded. Under the cell culture hood, the cerebral cortices were washed two times with pre-warmed PBS, minced with a scalpel and treated with 2 ml of pre-warmed trypsin-EDTA. After incubation at 37°C for 7 min with occasional shaking, trypsin was discarded and the cell clump washed two times with pre-warmed PBS. Cells were resuspended in Neurobasal medium (2.5 ml supplemented with 2% B27,

0.5 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin). Then cell clusters were separated into single cell suspension by triturating through a fire-polished Pasteur pipette.

Cells were counted using a hematocytometer and plated (200,000 cells in each well of a 24-well plate or 2,000,000 cells in each well of a 6-well plate coated with poly-D-lysine for pure cortical neurons or on a confluent glial cell layer for mixed neural cultures). Cultures were incubated at 37 °C under 5 % CO_2. To change medium, only half of medium was removed from each well and fresh pre-warmed medium was added.

For pure cortical neuronal cultures (neurons plated on poly-D-lysine coated plates), media was changed the first day after preparation (with full neurobasal medium containing 5 % FBS) and then every third day until cells were used for experiments at DIV 10 (Figure 2.1).

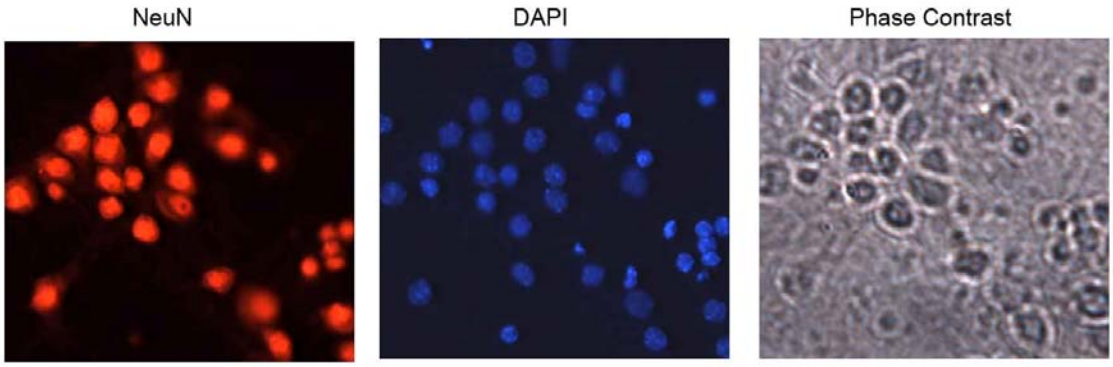


Figure 2.1 Immunohistochemistry (IHC) of NeuN in primary cortical neurons.

For mixed neural cultures (neurons plated on glial cell layer), media (full DMEM containing 25 % full neurobasal medium) was changed every 4 days and the cultures were used 6 days after plating neurons.

Coating plates with poly-D-lysine

Five hundred µl/well (in 24-well plate) or 2 ml/well (in 6-well plate) of poly-D-lysine solution (50 µg/ml in d H_2O) were added and the plates were incubated at 37 °C overnight then the solution was sucked off and the plates washed once with PBS and allowed to dry under the hood before neurons were plated.

2.1.4. Isolation of peritoneal macrophages

Peritoneal macrophages were prepared as described previously (L. Dory, 1989). Briefly, mice were killed by CO_2 inhalation. Then, peritoneal lavage using 10 ml ice cold PBS was performed 5 times. The collected solution was filtered through a 40 µm sieve and centrifuged at 1,000 x g for 10 min at 4 °C. The cell pellet was resuspended in 2 ml ACK (RBCs lysis) buffer and incubated at room temperature for 5 min before 10 ml PBS were added to stop the reaction. Then the cell suspension was centrifuged again at 1,000 x g for 10 min at 4 °C before the supernatant was discarded and the pellet resuspended in full DMEM medium containing 25 % neurobasal full medium. 400,000 cells were plated in each well of confluent mixed neural cultures in a 24-well plate (Figure 2.2).

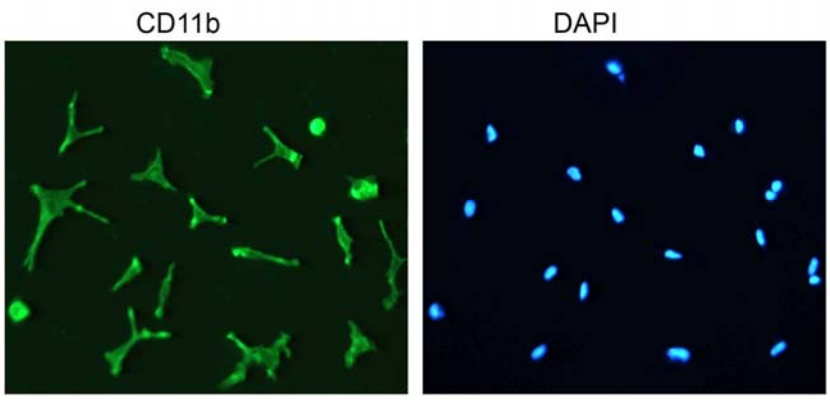


Figure 2.2 IHC of CD11b in peritoneal macrophages.

2.2. Oxygen Glucose Deprivation (OGD)

Materials:

2-Deoxy-D-Glucose	Sigma

OGD was used as an *in vitro* model of ischemia. Culture medium was replaced with stimulation medium (full neurobasal medium without B27 supplement) with (OGD plate) or without (control plate) 2-deoxy-D-glucose (5 mM). The plates were incubated at 37 °C for 45 min under normal conditions. Then the OGD plate was transferred to an anaerobic chamber, which was flushed with 5 % CO_2 and 95 % N_2 for 15 minutes before the anaerobic chamber was tightly sealed and placed in a normal incubator adjusted to 37 °C, while the control plate was kept under normal conditions (37 °C, 5 % CO_2). After the specified duration of OGD (4.5 h), medium was removed from both the control and the OGD plates and replaced with fresh stimulation medium. Both plates were incubated at 37 °C under normal conditions for 24 h before media were collected and RNA was extracted from the cells.

2.3. Quantification of cell death

Cell death can be classified into 2 main forms. Necrosis is accompanied by increased ion permeability of the plasma membrane associated with swelling and plasma membrane rupture. And apoptosis is characterized by condensation of nuclei and activation of endogenous nucleases which lead to DNA cleavage.

2.3.1. Quantification of cell death and lysis by measurement of lactate dehydrogenase (LDH) activity

The leakage of LDH through the damaged cell membrane is an indicator of plasma membrane damage and cell death. We quantified the released LDH and this was expressed relative to the control conditions.

Materials:

Cytotoxicity Detection Kit (LDH) — Roche

The kit reaction mix for 100 samples consisted of: 250 µl catalyst solution + 11.25 ml Dye solution (freshly mixed).

The cell culture medium was collected in Eppendorf tubes, spinned at 8,000 x g for 5 min at 4 °C to remove cells. Then, 100 µl medium were transferred into 96 well plate and mixed with 100 µl LDH kit reaction mix and incubated for 30 min at room temperature in the dark. The absorbance was then measured at 492 nm and reference 620 nm.

2.3.2. Detection of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes)

When a sample containing DNA-histone complexes (nucleosomes) is incubated with anti-histone (biotin-labeled) antibody and anti-DNA (peroxidase-conjugated) antibody in a streptavidin-coated microplate, the anti-histone biotin-labeled antibody binds the histone component of the nucleosomes and captures it to the streptavidin coat of the plate. Simultaneously, the peroxidase-conjugated anti-DNA reacts with the DNA component of the nucleosome and develops color using ABTS as a substrate (sandwich ELISA).

Materials:

Cell death detection ELISA ^{plus} — Roche

The kit is composed of the following
- Anti-Histone-Biotin (biotin-labelled monoclonal mouse antibody)
- Anti-DNA-POD (peroxidase conjugated monoclonal mouse antibody)
- Positive control (DNA-Histone-Complex)
- Incubation buffer
- Lysis buffer
- Substrate buffer

- ABTS substrate tablet (2,2-azino-di-3-ethylbenzthiazoline-sulfonic acid)
- ABTS stop solution
- Streptavidin-coated microplate

At the end of the stimulation period, the cell culture medium was collected and used for LDH measurement. The cells were washed once with PBS and lysis buffer (200 µl) was added to each well and incubated for 30 min at room temperature. Then cell lysates were collected into tubes and centrifuged at 200 x g for 10 min. Twenty µl of positive control, negative control, background control (incubation buffer) or clear supernatant were carefully transferred to the middle of the appropriate wells of the streptavidin-coated microplate. Freshly prepared immunoreagant (80 µl of 1 volume Anti-Histone-Biotin + 1 volume Anti-DNA-POD + 18 volume Incubation buffer) was added to each well and the plate was covered with adhesive cover foil and incubated at room temperature for 2 hours on a shaker (300 rpm/min).

After the incubation period, the solution was removed and the plate was washed 3-times each with 300 µl incubation buffer. Then ABTS solution (100 µl of 1 ABTS tablet dissolved in 5 ml substrate buffer) was added to each well and incubated on a shaker (250 rpm) for 10 – 20 min. Then ABTS stop solution (100 µl) was pipetted into each well. The signal was read at 405 nm and reference 490 nm and ABTS stop solution was used as a blank.

2.4. Quantification of Prostaglandin E_2 (PGE_2) release.

Materials:

Prostaglandin E_2 EIA kit-monoclonal Cayman

The kit is composed of the following
- Prostaglandin E_2 Monoclonal antibody
- Prostaglandin E_2 AchE Tracer
- Prostaglandin E_2 EIA Standard
- EIA Buffer
- Wash Buffer
- Goat Anti-Mouse IgG Coated plate

- Ellman's Reagant

The cell culture medium was collected in Eppendorf tubes and centrifuged at 8,000 x g for 5 min at 4 °C to remove cells. Then medium or standard (50 µl) was transferred into the appropriate wells and PGE_2 AChE tracer and PGE_2 monoclonal antibody (50 µl each) were added. After 18 h incubation at 4 °C, the plate was washed and Ellman's reagent (200 µl) was added to each well and absorbance was measured at 420 nm after 60 min.

2.5. RNA extraction and reverse transcription

Materials:

RNApure		Peqlab
Chloroform		Sigma
Isopropanol		J.T.Baker
70 % Ethanol		J.T.Baker
5 x Buffer	10 µl	Promega
dNTPs (25 mM)	2 µl	Promega
Random Primer	1 µl	Promega
RNAsin	1 µl	Promega
Reverse Transcriptase	1 µl	Promega

2.5.1. RNA extraction from cultured cells

Cell culture medium was removed and the cells were washed once with cold PBS. Then RNApure reagent was added (1 ml/well in 6-well plate) and cells were triturated several times. Cells were allowed to stand on ice for 7 min before the cell lysate was transferred to Eppendorf tubes containing 0.2 ml chloroform. Lysates were mixed for 15 seconds and again allowed to stand for 7 min on ice. Samples were then centrifuged at 13,000 x g for 20 min at 4 ° C. The upper aqueous layer containing RNA was carefully transferred to another tube containing 0.5 ml isopropanol and incubated overnight at – 20 °C to allow for the precipitation of RNA. Samples were then centrifuged at 13,000 x g for 20 min at 4 ° C and the RNA pellet was washed two times with 1 ml 75 % ethanol in water. The pellet was allowed to dry partially at room temperature and then dissolved in dH_2O. The RNA

concentration was measured using a spectrophotometer. RNA was stored at – 80 °C.

2.5.2. RNA extraction from brain tissue

Fifty mg homogenized tissue from the ischemic core or the surrounding periphery were used for the extraction of RNA using the same protocol as described with cultured cells.

2.5.3. Reverse Transcription

Three µg RNA from each sample were diluted to a final volume of 35 µl with dH_2O. Then RNA was mixed with the above-mentioned volumes of 5X buffer, dNTPs, random primer, RNasin and reverse transcriptase and incubated at room temperature for 10 min and at 37 °C for 90 min. The obtained cDNA was stored at – 20 °C.

2.6. Polymerase chain reaction (PCR), Reverse transcription-PCR (RT-PCR) and Real-Time RT-PCR

2.6.1. Real time RT-PCR

The expression of the target gene(s) was quantified relative to the expression of a house keeping gene (cyclophillin) under the different experimental conditions using real-time RT-PCR

Materials:

Real-Time RT-PCR Cycler: Gene Amp 5700 Sequence Detector, PE	Applied Biosystems
SYBR Green Kit	PE Applied Biosystems
The reaction mix was composed of	
Master mix (SYBRGreen) 15 µl	
Primer 1 (5 µM) 1.8 µl	
Primer 2 (5 µM) 1.8 µl	
dH_2O 6.4 µl	
PCR plates	ABgene
Primers were synthesized by	TIB-MOLBIOL

Primer	Primer sequence	Amplicon length
Cyclophilin-F	5' AGG TCC TGG CAT CTT GTC CAT 3'	51 bp
Cyclophilin-R	5' GAA CCG TTT GTG TTT GGT CCA 3'	
cPLA-2-F	5' TTG GCG ATA TGC TGG ACA CTC 3'	84 bp
cPLA-2-R	5' AGT GTC TCG TTC GCT TCC TGCT 3'	
COX-2-F	5' CAG ACA ACA TAA ACT GCG CCT T	71 bp
COX-2-R	5' GAT ACA CCT CTC CAC CAA TGA CC	
mPGES-1-F	5' AAG ATG TAC GCG GTG GCT GTC A	55 bp
mPGES-1-R	5' AAG CCT TCT TCC GCA GCC TCA T 3'	

cDNA samples were diluted 1:16 for cPLA-2, COX-2 and mPGES-1 or 1:1024 for cyclophillin and 5 µl were mixed with the above-mentioned volumes of the master mix, primers and dH_2O in a real-time RT-PCR plate. The real-time RT-PCR was performed according to the following protocol:

10 minutes at 95 °C
15 seconds at 95 °C
1 minute at 60 °C

Repeated for 40 cycles

A linear dilution-amplification curve was obtained from diluted pooled samples (1:2 till 1:64) for cPLA-2, COX-2, mPGES-1 or (1:128 till 1: 4096) for cyclophillin. Using this curve the expression of each gene was quantified relative to the expression of the house keeping gene under different experimental conditions.

The purity of the amplified products was checked by the dissociation curve and by gel electrophoresis.

2.6.2. RT-PCR

Materials:

Taq polymerase — Taqara
dNTPs — Promega
TBE buffer (Tris/Borate/EDTA)

Tris-borate	89 mM
EDTA	2 mM

Agarose	Biozyme
Primers were synthesized by	TIB-MOLBIOL

Primer	**Primer sequence**	**Amplicon length**
TLR-2-F	5' AGG CTC GGT TCT CAC TGA TGA A 3'	623 bp
TLR-2-R	5' CTA ACA TCC AAC ACC TCC AGC G 3'	
TLR-4-F	5' GAC ACC AGG AAG CTT GAA TCC 3'	603 bp
TLR-4-R	5' GGC TTG GTC TTG AAT GAA GTC A 3'	
RAGE-F	5' GAT TCC CGA TGG CAA AGA AAC AC 3'	512 bp
RAGE-R	5' ACT CAC CCA CAG AGC CTT CAG 3'	
GAPDH-F	5' ATC CTG CAC CAC CAA CTG CTT A 3'	711 bp
GAPDH-R	5' TTC AAG AGA GTA GGG AGG GCT 3'	

To investigate the presence of HMGB1 receptors (TLR-2, -4 and RAGE) in our cell culture model, we performed RT-PCRs using cDNA obtained from spleen, whole brain, cultured neurons or cultured microglia. For comparison, levels of the house keeping gene GAPDH were determined using the following PCR reaction:

Taq polymerase	0.25 µl
dNTPs	4 µl
10 x buffer	5 µl
Primer F (10 µM)	5 µl
Primer R (10 µM)	5 µl
cDNA	7 µl
water to	50 µl

and the PCR protocol consisted of the following steps:

Initial denaturation	94 °C	1 min
Denaturation	94 °C	15 sec
Annealing	* °C	30 sec
Extension	72 °C	1 minute
		Repeated 40 x
Final extension	72 °C	7 minute
Hold	4 °C	

* The annealing temperature was adjusted for each PCR product according to the primers used:

TLR-2	56 °C
TLR-4	53 °C
RAGE	52 °C
GAPDH	61 °C

2.6.3. Genotyping of CD11b-DTR mice

Materials:

Non Ionic detergent (NID) buffer:

50 mM HCl	J.T Baker
10 mM Tris/Cl pH 8.3	Roth
2 mM $MgCl_2$	Fluka
0.1 mg/ml gelatin	Gruessing
0.45 % NP40	
0.45 % Tween 20	Roth
Proteinase K	Merck
Taq polymerase	Taqara
Primers were synthesized by	TIB-MOLBIOL

Primer	**Primer sequence**	**Amplicon length**
WT-F	5’ CTA GGC CAC AGA ATT GAA AGA TCT 3’	324 bp
WT-R	5’ GTA GGT GGA AAT TCT AGA ATC ATC C 3’	
TG-F	5’ GAG GGC GAT GCC ACC TAC GGC AAG 3’	500 bp
TG-R	5’ CTA AGG GCG GAC TGG GTG CTC AGG 3’	

At the age of P2, heterozygous CD11b-DTR mice were killed by CO_2 inhalation. A part of the tail from each mouse was incubated in 200 µl NID buffer and 2 µl proteinase K (10 mg/ml) at 56 °C for 6 hours with mild shaking. Then proteinase K was inactivated by heating at 95 °C for 10 min. The obtained genomic DNA was used to perform PCR reaction for the transgene (TG) and the wild-type (WT) allele as an internal standard using the following procedure:

WT

The PCR reaction contained

$MgCl_2$	1.5 µl
dNTPs (5 mM)	0.4 µl
10x buffer	5 µl
WT-F (10 µM)	3 µl
WT-R (10 µM)	3 µl
DNA	1.3 µl
Taq polymerase	0.2 µl
dH_2O	35.6 µl

and the PCR protocol consisted of the following steps:

Initial denaturation	94 °C	4 min	
Denaturation	94 °C	30 sec	
Annealing	60 °C	30 sec	
Extension	72 °C	30 sec	
			Repeated 32 x
Final extension		72 °C	5 minute
Hold	4 °C		

TG

The PCR reaction contained

$MgCl_2$	2 µl
dNTPs (5 mM)	0.4 µl
10x buffer	5 µl
WT-F (10 µM)	3 µl
WT-R (10 µM)	3 µl
DNA	1 µl
Taq	0.2 µl
dH_2O	35.4 µl

and the PCR protocol consisted of the following steps:

Initial denaturation	94 °C	4 min
Denaturation	94 °C	45 sec
Annealing	64 °C	45 sec
Extension	72 °C	30 sec
		Repeated 32 x
Final extension	72 °C	5 minute
Hold	4 °C	

The PCR products were ran on an agarose gel (1% in 0.5 x TBE) next to a marker (Figure 2.3).

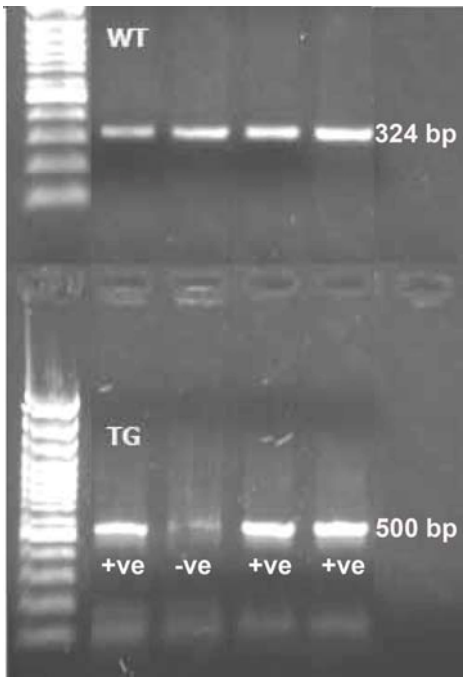


Figure 2.3 Genotyping of CD11b-DTR mice.

2.7. Immunohistochemistry

Materials:

Para–formaldehyde (PFA)	Sigma
Triton 100-X	Merck
Tween 20	Roth
Normal goat serum (NGS)	Vector Laboratories
Normal horse serum (NHS)	Vector Laboratories
Mouse anti-NeuN antibody	Chemicon international
Mouse anti-GFAP	Santa cruz
Rabbit anti-IBA-1 antibody	Wako
Rabbit polyclonal anti-HMGB1 antibody	
Rat anti-CD11b	Serotec
Goat anti-RAGE antibody	Biologo
Rhodamine (TRITC) goat anti-mouse antibody	Jackson Laboratories
Alexa Fluor 488-conjugated donkey anti-mouse antibody	Invitrogen
Fluorescein-conjugated anti-rabbit antibody	Vector Laboratories
Cy3-conjugated goat anti-rabbit antibody	Jackson
Alexa Fluor 488-conjugated donkey anti-rat antibody	Invitrogen
Cy3-conjugated donkey anti-goat antibody	Jackson
DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride)	Sigma
Mowiol	Calbiochem

2.7.1. Anti-Neuronal Nuclei (NeuN) Staining

After removal of cell culture medium, the cells were fixed with 4 % PFA for 30 minutes, washed with PBS before they were permeabilised for 5 min in Triton 0.25 % and Tween 0.1 % for another 5 minutes. The cells were then washed with PBS and blocked with 5 % normal goat serum (NGS) in PBS for 1 hour at room temperature. Then, cells were incubated with the primary mouse anti-NeuN antibody (1:50 in 5% NGS) for 1 hour at room temperature. The cells were then washed with PBS and incubated for 30 min with the secondary antibody (Rhodamine [TRITC] goat anti-mouse antibody, 1:100) at room temperature and protected from light. Finally the cells were treated with DAPI in water (1:10,000) for 5 min, washed with water and mounted with Mowiol DAPCO.

2.7.2. Anti-Glial Fibrillary Acidic Protein (GFAP) Staining

After removal of cell culture medium, the cells were fixed with 4 % PFA for 30 minutes, washed with PBS before they were permeabilised for 5 min in Triton 0.25 % and Tween 0.1 % for another 5 minutes. The cells were then washed with PBS and blocked with 5 % normal goat serum (NGS) in PBS for 1 hour at room temperature. Then, cells were incubated with the primary mouse anti-GFAP antibody (1:50 in NGS) for 1 hour at room temperature. The cells were then washed with PBS and incubated for 30 min with the secondary antibody (alexa fluor 488-conjugated donkey anti-mouse, 1:100) the secondary antibody (Rhodamine [TRITC] goat anti-mouse antibody, 1:100) at room temperature and protected from light. Finally the cells were treated with DAPI in water (1:10,000) for 5 min, washed with water and mounted with Mowiol DAPCO.

2.7.3. Anti- Ionized calcium binding adaptor molecule 1 (Iba-1) Staining

After removal of cell culture medium, the cells were fixed with 4 % PFA for 30 minutes, washed with PBS before they were permeabilised for 5 min in Triton 0.25 % and Tween 0.1 % for another 5 minutes. The cells were then washed with PBS and blocked with 5 % normal goat serum (NGS) in PBS for 1 hour at room temperature. Then, cells were incubated with the primary rabbit anti-IBA-1 antibody

(1:1000 in NGS) for 1 hour at room temperature. The cells were then washed with PBS and incubated for 30 min with the secondary antibody (flourescein-conjugated anti-rabbit antibody, 1:100) at room temperature and protected from light. Finally the cells were treated with DAPI in water (1:10,000) for 5 min, washed with water and mounted with Mowiol DAPCO.

2.7.4. Anti-High Mobility Group Box-1 protein (HMGB1) Staining

After removal of cell culture medium, the cells were fixed with 4 % PFA for 30 minutes, washed with PBS before they were permeabilised for 5 min in Triton 0.25 % and Tween 0.1 % for another 5 minutes. The cells were then washed with PBS and blocked with 5 % normal horse serum (NHS) in PBS for 1 hour at room temperature. Then, cells were incubated with the primary rabbit polyclonal anti-HMGB-1 antibody (which was kindly provided by Dr K. J: Tracey, Feinstein Institute for Medical Research, Manhasset, USA), the anti-HMGB1 antibody was applied in a dilution of 1:200 in NHS for 1 hour at room temperature. The cells were then washed with PBS and incubated for 30 min with the secondary antibody (Cy3-conjugated goat anti-rabbit antibody, 1:100) at room temperature and protected from light. Finally the cells were treated with DAPI in water (1:10,000) for 5 min, washed with water and mounted with Mowiol DAPCO.

2.7.5. Anti- Cluster of Differentiation molecule 11b (CD11b) Staining

After removal of cell culture medium, the cells were fixed with 4 % PFA for 30 minutes, washed with PBS before they were permeabilised for 5 min in Triton 0.25 % and Tween 0.1 % for another 5 minutes. The cells were then washed with PBS and blocked with 5 % normal horse serum (NHS) in PBS for 1 hour at room temperature. Then, cells were incubated with the primary rat anti-CD11b antibody (1:200 in NHS) for 1 hour at room temperature. The cells were then washed with PBS and incubated for 30 min with the secondary antibody (Alexa Fluor 488-conjugated donkey anti-rat antibody, 1:100) at room temperature and protected from light. Finally the cells were treated with DAPI in water (1:10,000) for 5 min, washed with water and mounted with Mowiol DAPCO.

2.7.6. Anti-Receptor for Advanced Glycation Endproducts (RAGE) Staining

After removal of cell culture medium, the cells were fixed with 4 % PFA for 30 minutes, washed with PBS before they were permeabilised for 5 min in Triton 0.25 % and Tween 0.1 % for another 5 minutes. The cells were then washed with PBS and blocked with 5 % normal horse serum (NHS) in PBS for 1 hour at room temperature. Then, cells were incubated with the primary goat anti-RAGE antibody (1:200 in NHS) for 1 hour at room temperature. The cells were then washed with PBS and incubated for 30 min with secondary antibody (Cy3-conjugated donkey anti-goat antibody, 1:100) at room temperature and protected from light. Finally the cells were treated with DAPI in water (1:10,000) for 5 min, washed with water and mounted with Mowiol DAPCO.

2.8. Cloning

Materials:

Phusion Polymerase	Finnzymes
dNTPs	Finnzymes
Primers were synthesized by	TIB MOLBIOL

Primer	Extra bases	Res. Enz. Rec. Seq.	Main sequence	Amplicon length
cPLA2-1750-F	5'ATTCA	GGATCC	TTCAAACCCTGCAGTGCCT	1830 bp
cPLA2-900-F	5'ATTCA	GGATCC	AGGCATTCTAACCAGGGTAC	980 bp
cPLA2-R	5'TCGTA	CTCGAG	TGAGAATCCTCAGGCTTCTC	
COX2-1750-F	5'ATTCA	GGATCC	GAGGATGGAGTTGGTCAAAGTC	1830 bp
COX2-900-F	5'ATTCA	GGATCC	GGTTAGGGAGAATAAGGCTAGT	980 bp
COX2-R	5'TCGTA	CTCGAG	AGTAGTGGTGGCGGTGGAGC	

TAE buffer (Tris/Acetate/EDTA)
Tris-acetate (40 mM)
EDTA (1 mM)

QIAquick PCR purification kit supplied with the following solutions: Buffer PBI Buffer PE Buffer EB	Qiagen
BamHI XhoI Buffer B (10x) BSA	Promega Promega Promega BioLabs
QIAquick gel extraction supplied with the following solutions: Buffer QG Buffer PE Buffer EB	Qiagen
Ligation Buffer T4 DNA Ligase	Roche Roche
Gene Ruler 1 κB DNA ladder $CaCl_2$ (0.1 M)	Fermentas Merck
LB Medium (Lysogeny broth) * 1 % Peptone from Casein 0.5 % Yeast extract 0.5 % NaCl	 Fluka Roth Prolabo
HB101 competent cells	Promega
SOC medium (Super Optimal broth, SOB, with Catabolite repression) * 2 % Peptone from casein 0.5 % Yeast extract 20 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM Glucose	 Fluka Roth Prolabo Merck Merck J.T.Baker Merck
Agar	GibcoBRL
Ampicillin	Roth

Agar plates 1.5 % agar in LB + 50 µg/ml ampicillin

Taq Polymerase — Taqara

PureYield Plasmid Midiprep System supplied with the following reagents — Promega

- Cell Resuspension solution (CRA)
  50 mM Tris-HCL, pH 7.5
  10 mM EDTA, pH 8.0
  100 µg/ml RNase A

- Cell Lysis Solution (CLA)
  0.2 M NaOH
  1 % SDS

- Neutralisation solution (NSB)
  4.09 M Guanidine HCl (pH 4.8)
  759 mM Potassium Acetate
  2.12 M glacial acetic acid

- Endotoxin Removal wash
  162.8 mM potassium acetate
  22.6 mM Tris-HCl (pH 7.5)
  0.109 mM EDTA (pH 8.0)
  supplemented with 95 % ethanol before use

- Column Wash

Plasmid purification maxi kit supplied with the following reagents — Qiagen

- Resuspension Buffer (P1)
  50 mM Tris-HCL, pH 8.0
  10 mM EDTA
  100 µg/ml RNase A

- Lysis Buffer (P2)
200 mM NaOH
1 % SDS
- Neutralisation Buffer
3 M Potassium Acetate, pH 5.5
- Equilibration buffer (QBT)
750 mM NaCl
50 mM MOPS, pH 7.0
15 % isopropanol (v/v)
0.15 % Triton X-100 (v/v)
- Washing Buffer (QC)
1 M NaCl
50 mM MOPS, pH 7.0
15 % isopropanol (v/v)
- Elution Buffer (QF)
1.25 M NaCl
50 mM Tris-HCl, pH 8.5
15 % isopropanol (v/v)

BD+ (BigDye® Terminator v1.1 Cycle Sequencing Kit)	Applied Biosystems
Luc-5 primer 5' CCATTT TACCAA CAGTAC CG	TIB MOLBIOL
Sephadex	GE Health Care Biosciences
Hi-Di™ Formamide	Applied Biosystems

* LB and SOC were sterilized by autoclaving and ampicillin was added to LB after the solution had reached a temperature of about 50 – 55 °C.

The promoter region (-2000 base downstream and +200 b upstream of the transcription start site TSS) for cPLA-2, COX-2 and mPGES-1 were analysed to find the probable transcription factor binding sequences in that region using an online software (http://www.gene-regulation.com/).

Interestingly, we found several sites where NF-κB can bind in the promoter regions for these genes and we decided to prepare two constructs for each gene, a long

one starting at –1750 to +80 relative to the TSS and a short construct starting at –900 to +80 relative to the TSS (of cPLA-2 and COX-2) and cloned them into the promoterless vector pXP2. PCR primers producing these fragments were designed in a way that the 5' end of each primer contained - in addition to the required DNA sequence – a recognition sequence for the restriction enzymes BamHI (GGATCC in case of the forward primer) and XhoI (GAGCT in case of the reverse primer) preceded by 5 non-specific bases to ensure that the restriction enzyme will be able to cut at that site.

For mPGES-1, constructs containing the sequences –1814 to +33 and –930 to +33 in the promoterless vector pGL3-basic were kindly provided by Dr. Hiroaki Naraba (Iwate Medical University, Japan) and were described previously in details (H. Naraba et al., 2002).

2.8.1. Cloning PCR

The PCR reaction consisted of

buffer (5x)	10 µl
dNTPs (5mM)	2 µl
primer F (10 µM)	1 µl
primer R (10 µM)	1 µl
Phusion Polymerase	0.5 µl
genomic DNA	2 µl
dH_2O	33.50 µl

The following PCR protocol was used

Initial denaturation	98 °C	30 seconds
Denaturation	98 °C	10 seconds
Annealing	60 °C	30 seconds for 1 κB 1 minute for 2 κB
Extension	72 °C	1 minute Repeated 30 x
Final extension	72 °C	7 minute
Hold	4°C	

Then, the PCR products were ran on an agarose gel (1% in 0.5 x TAE) (Figure 2.4) and purified using the PCR product purification kit (Qiagen).

2.8.2. PCR product purification

The PCR product (100 µl) was mixed with 500 µl buffer PBI and applied to the spin column assembled in a collection tube. Centrifugation at 10,000 x g for 1 min at room temperature allowed DNA to bind to the column. Then, the flow-through was discarded and the column was washed with 750 µl buffer PE. After centrifugation for 1 min the flow-through was discarded and the column centrifuged again for 1 min.

The column was removed to a new 1.5 ml tube, buffer EB (50 µl) was added to the center of the membrane and the column was centrifuged for 1 min to elute DNA.

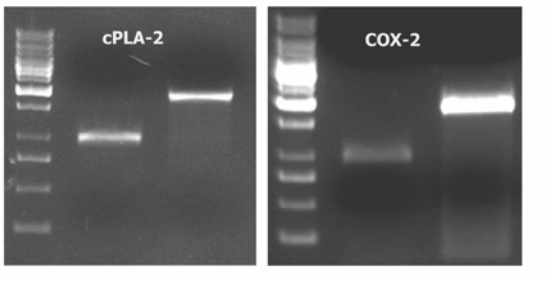


Figure 2.4 cPLA-2 and COX-2 constructs

2.8.3. Cleavage with restriction enzymes

Both the DNA insert and vector were cleaved by the restriction enzymes BamHI and XhoI

Vector (pXP2) cleavage

Buffer (10x)		2 µl
Acetylated BSA	(10 µg/µl)	0.2 µl
Plasmid (1µg)		5 µl
dH_2O		12.3 µl
BamHI		0.5 µl
XhoI		0.5 µl

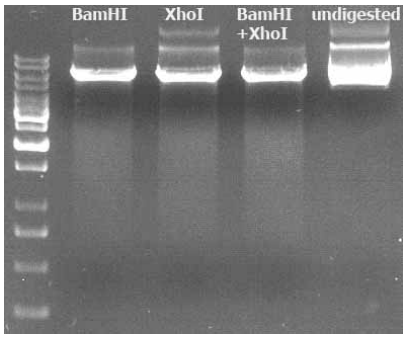


Figure 2.5 pXP2 linearized by the restriction enzymes and subjected to agarose electrophoresis

Insert cleavage

Buffer (10x)	2 µl
Acetylated BSA (10 µg/µl)	0.2 µl
DNA	18 µl
BamHI	0.5 µl
XhoI	0.5 µl

After cleavage (4 h at 37 °C), 4 µl loading buffer was added and the cut product was loaded on 1 % agarose gel in TAE to check again for the correct size (Figure 2.5).Then the bands were cut and purified using the Gel purification kit (Qiagen).

2.8.4. Gel purification

Gel fragments containing the DNA fragment of the correct size were excised by a clean scalpel and weighed. Buffer QG was added (in a volume equal to 3 times the gel weight) and incubated at 50 °C for 10 min till the gel was dissolved. Then isopropanol was added (in equal volume to the gel weight) and the mixture was applied into a spin column assembled in a collection tube. The column was centrifuged at 10,000 x g for 1 min at room temperature to allow DNA to bind to the column.

The flow-through was discarded and Buffer QG (500 µl) was added and the column was centrifuged for 1 min to remove any traces of agarose before the column was washed with 750 µl buffer PE and centrifuged for 1 min.

The flow-through was discarded and the column was centrifuged for additional 1 min. The column was removed to a new 1.5 ml tube and 50 µl buffer EB added to the center of the membrane and centrifuged for 1 min to elute DNA.

2.8.5. Ligation

The concentration of digested vector and DNA insert was estimated by comparing the band intensity on the gel with that on the product data sheet of the ladder (Gene Ruler).

In each ligation reaction, a total of 1 µg DNA (vector: insert in a ratio of 1:2) was mixed with 3 µl ligation buffer, 2 µl T4 DNA Ligase and dH_2O to 30 µl. The reaction was incubated overnight at 4 °C and the ligated product was used to transform competent cells.

2.8.6. Generation of Competent HB101 cells

Commercially available HB101 cells were streaked on the surface of an agar plate without antibiotics and grown overnight at 37 °C. Then, a single colony was picked and inoculated into 3 ml LB medium free from antibiotics and grown overnight at 37 °C with shaking. This bacterial suspension was used to inoculate a larger volume of LB without antibiotics (200 ml) and incubated at 37 °C with shaking until they reached the logarithmic growth phase (Optical Density OD_{600} = 0.4 – 0.6). The bacterial suspension was then centrifuged at 5,000 x g for 5 min at 4 °C and the pellet was resuspended in 200 ml ice-cold 0.1 M $CaCl_2$ and again centrifuged at 5,000 x g for 5 min at 4 °C. Washing with $CaCl_2$ was repeated two times before the pellet was resuspended in 5 ml ice-cold $CaCl_2$ and 100 µl aliquots were transferred to pre-chilled Eppendorf tubes. The samples were frozen in liquid nitrogen and stored at – 80 °C. To test for absence of any resistance to antibiotics, competent cells were streaked on the surface of an agar plate containing ampicillin on which they did not grow after overnight incubation at 37 °C. Also the competence of the cells was evaluated by test transformation using a control plasmid (HB101 competent cells have no resistance to antibiotics and they gain resistance

against certain antibiotic when they take in a plasmid expressing resistance gene to antibiotics, e.g. ampicillin in case of pXP2 or pGL3-basic).

2.8.7. Transformation

HB101 cells were transformed with the blank vector (pXP2 or pGL3-basic) or vectors containing the DNA inserts as follows:

100 µl competent cells (HB101)
+ 2 µl vector,
on ice for 5 minutes
at 42°C for 50 seconds
on ice for 2 minutes

Then 900 µl LB medium or SOC were added to the mixture and incubated at 37 °C for 1 hr with shaking (900 rpm).

The bacteria/vector solution (100 µl) was streaked on the surface of an agar plate containing ampicillin (50 µg/ml) so that only bacterial cells containing the vector could grow. In addition, the remaining solution was centrifuged at 5,000 x g for 5 minutes at 16 °C. The supernatant was discarded, the pellet was resuspended in 100 µl SOC and plated on another agar plate containing ampicillin. The plates were incubated inverted up-side-down overnight at 37 °C.

To ensure appropriate ligation of the vector and the DNA insert in bacterial colonies that grew on ampicillin plates after transformation, colony PCR was performed to detect the presence of the correct DNA insert in several single colonies. Several single colonies were picked and each resuspended in 20 µl water before colony PCR was performed.

2.8.8. Colony PCR

The PCR reaction consisted of

buffer (10x)	5 µl
dNTPs (5 mM)	1 µl
primer F (10 µM)	2.5 µl
primer R (10 µM)	2.5 µl
Taq polymerase	1 µl
DNA	10 µl
d H_2O	28 µl

The following PCR protocol was used

Initial denaturation	94 °C	10 minutes
Denaturation	94 °C	20 seconds
Annealing	58 °C	30 seconds
Extension	72 °C	2 minute
		repeated 30 x
Final extension	72 °C	7 minute
Hold	4°C	

Then, a positive colony (containing the correct insert size) (Figure 2.6) was inoculated in 3 LB (+ ampicillin 50 µg/ml) and grown overday (8 h) and then used to inoculate larger volumes of LB (+ ampicillin) which was used for the isolation of large amounts of the plasmid.

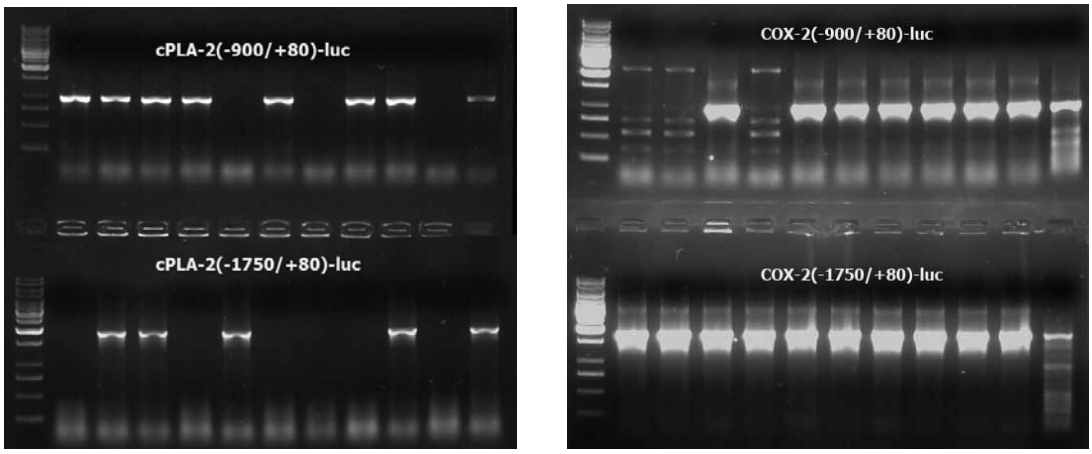


Figure 2.6 Colony PCR for cPLA-2 and COX-2 constructs

2.8.8. Purification of the plasmid

For purification of the plasmids we used PureYield Plasmid Midiprep System (Promega) or Qiagen plasmid purification maxi kit.

2.8.8.1. PureYield Plasmid Midiprep System (Promega)

Thirty µl bacterial suspension were inoculated in 3 ml LB (containing ampicillin 50 µg/ml) and incubated at 37 °C with shaking for 8 hours. Then, 100 µl of the bacterial suspension were inoculated into 100 ml LB (+ amp) in a 500-ml sterile flask and incubated at 37 °C with shaking for 16-21 h.

The cells were collected by centrifugation at 4.000 x g for 10 min at 16 °C and then resuspended in 3 ml cell resuspension solution. Cell lysis solution (3 ml) was added and mixed by inverting 5 times. After 3 min incubation at room temperature, neutralisation solution (5 ml) was added. The solution was mixed by inverting 10 times and incubated for 3 min at room temperature. Finally it was centrifuged at 6,000 x g for 30 min at 16 °C.

The supernatant was carefully transferred into the blue clearing column assembled on the white binding column connected to a vacuum manifold. The supernatant was left for 2 min to allow the debris to float upwards and then vacuum was applied till the solution passed through both columns. Then, the vacuum was released and the blue column discarded before 5 ml endotoxin removal wash were applied and allowed to pass through the column.

Twenty ml column wash were added and allowed to pass through the column. The vacuum continued for another 1-2 min to dry the column. Then, the column was transferred to a 50-ml tube and 600 µl nuclease free water were added. To elute DNA, after 5 min at room temperature, the tubes (without lid) were centrifuged at 2,000 x g for 5 min at 16 °C. The concentration of the eluted DNA was determined spectrophotometrically at 260 nm.

2.8.8.2. Plasmid purification maxi kit (Qiagen)

Thirty µl bacterial suspension were inoculated in 3 ml LB (containing ampicillin 50 µg/ml) and incubated at 37 °C with shaking for 8 hours. Then, 200 µl of the bacterial suspension were inoculated into 100 ml LB (+ amp) in a 500-ml sterile flask and incubated at 37 °C with shaking for 12-16 h.

The cells were collected by centrifugation at 6,000 x g for 15 min at 4 °C and then resuspended in 10 ml resuspension buffer P1 supplemented with RNase A (DNase free). Ten ml lysis buffer P2 were added and mixed by inverting 5 times. After 5 min incubation at room temperature, 10 ml of pre-chilled neutralisation buffer P3 were added. The solution was mixed by inverting 5 times and incubated for 20 min on ice. After further mixing, it was centrifuged at 14,000 x g for 30 min at 4 °C.

The supernatant was carefully transferred into a new tube and centrifuged again at 14,000 for 15 min at 4 °C. Then, the supernatant was carefully transferred into a column which was previously equilibrated with 10 ml equilibration buffer QBT. The solution was allowed to drain through the column by gravity and then the column was washed two times each with 30 ml wash buffer QC. DNA was eluted into a new tube using 15 ml elution buffer GF and then precipitated with 10.5 ml isopropanol. After centrifugation at 14,000 x g for 15 min at 4 °C the DNA pellet was washed with 5 ml 70 % ethanol, air dried and dissolved in sterile water. The concentration was determined spectrophotometrically at 260 nm

2.8.9. Sequencing

To ensure that PCR has synthesized the correct DNA insert(s) and that the previous steps did not cause any mutations in these sequences, we sequenced the DNA insert in each of our vectors.

The PCR reaction consisted of

BD+	3.2 µl
Primer (10 µM)	1.3 µl
Plasmid (1 µg)	
Water to 8 µl	

The following PCR protocol was used

94 °C	15 seconds
60 °C	4 minutes
	repeated 32 x

We performed two sequencing reactions for each insert using either the reverse primer used to synthesize that sequence or a primer directed against a sequence located in the plasmid (LUC-5) to get as much information as possible about the sequence of each insert.

Sephadex solution (750 µl) was applied to a separating column placed in a collection tube and centrifuged at 4,000 x g for 5 minutes. The flow-through was discarded before centrifuging again. Then, the column was placed in a new 1.5 ml tube and the PCR product (8 µl) + 2 µl water were applied to the column and centrifuged at 4,000 x g for 5 minutes.

The flow-through was collected and mixed with 10 µl HiDi in another 0.5 ml tube. Then the sequence was determined using the ABI Prism 310 Genetic Analyser. The obtained sequence was aligned to the original sequence of the DNA insert to check for any possible mutations or mismatches. Sequences were found to be identical using sequence analyser ABI prism software.

2.9. Transfection

Materials:

Lipofectamine 2000	Invitrogen
Opti-MEM I medium	Invitrogen
phRL-TK	Promega
pNF-κB-luc	Stratagene

Pure cortical neurons in (24 well plates) were transfected at DIV 10 as follows:

2.9.1. Transfection of a single plasmid

Cell culture medium was replaced with 500 µl transfection medium (full neurobasal medium without antibiotics) and incubated for 24 h. Then the blank plasmid or

plasmid containing the gene constructs (1 µg DNA/well) was diluted in 50 µl Opti-MEM I medium. Also 1.5 µl/well Lipofectamine 2000 was diluted in 50 µl Opti-MEM I and incubated for 5 minutes at room temperature. Then, the diluted DNA and Lipofectamine were combined, mixed gently and incubated for 20 minutes at room temperature. 100 µl of the combined solution were added to the appropriate wells (containing media without antibiotic) and mixed gently by rocking the plate. After incubation at 37 °C for 18-24 hours, the medium was replaced with stimulation medium with or without TNF and incubated for 6 hours before the transcriptional activity was assessed by luciferase assays.

2.9.2. Transfection of multiple plasmids

Plasmids containing the gene constructs (0.5 µg), pBS and p65 or Flag-IKK-2-EE (0.5 µg) together with 0.05 µg phRL-TK were transfected using the same protocol described above. However, the transcriptional activity was assessed by the dual luciferase assay.

In another experiment, neurons were transfected with phRL-TK and pNF-κB-luc for 18-24 hr. Then, the cells were subjected to OGD for 4.5 h and 24 h recovery, before the transcriptional activity was assessed by dual luciferase.

2.10. Luciferase Assay

Materials:

100 mM KH_2PO_4 (pH 7.8)	Merck
180 mM EGTA (pH7.4)	Roth
1 M $MgSO_4$	J.T.Baker
0.5 Glycylglycin (pH7.8)	Serva
DL-Dithiothreitol (DTT)	Roth
Luciferin	Sigma

Glycylglycin buffer (pH 7.8)

5 ml 0.5 M Glycylglycin
1.5 ml 1M $MgSO_4$
2.22 ml 180 mM EGTA
Complete with water to 100 ml

Assay Mix for 10 samples
- 1.5 ml Glycylglycin buffer
- 300 µl 100 mM KH_2PO_4
- 20 µl 100 mM DTT
- 4 µl 1M ATP.
- 16 µl water.

Extraction buffer for 10 samples
- 1.1 ml Glygylglycin buffer
- 11 µl Triton x-100 (1%)
- 11 µl 100 mM DTT (1mM)

Luciferin
- 55 mg DTT
- 35.7 ml Glycylglycin buffer on ice
- 10 mg luciferin
- store aliquots at – 80 °C.

Luciferin mix
- 600 µl luciferin
- 2.4 ml Glycylglycin buffer
- 240 µl 100 mM DTT.

The cell culture medium was removed and the cells were washed once with ice-cold PBS. Then extraction buffer (110 µl) was applied to each well and the cells were scraped and transferred to Eppendorf tubes. After centrifugation at 13,000 x g for 5 minutes at 4 °C, assay mix (184 µl) was applied in each measurement tube, and 50 µl sample were added just before measuring. The luminometer (Lumat LB 9501, Berthold) automatically subtracted the background activity, injected 100 µl luciferin solution and measured the activity in the sample.

2.11. Dual Luciferase Assay

Materials:

Dual Luciferase Assay Kit Promega

The kit is composed of:
- Passive lysis buffer
- LAR II
- Stop & Glo solution

The cell culture medium was removed and the cells were washed once with cold PBS. Then, passive lysis buffer (100 µl) was applied to each well and the cells were scraped and transferred to Eppendorf tubes. After centrifugation at 13,000 for 5 minutes at 4 °C, LAR II solution (100 µl) was applied in each measurement tube. The background activity was measured before the sample (20 µl) was added and the firefly luciferase activity measured. Finally, 100 µl Stop & Glo solution was added to stop the firefly luciferase activity and to start the renilla luciferase activity. Firefly luciferase activity was expressed relative to the renilla luciferase activity as a control for differences in transfection efficiency or cell death.

2.12. Statistics

Student t-test was used for comparison of two groups and one way ANOVA was used for comparison between more than two groups. Data were expressed as means ± S.E.M.

3. Results

Ischemia is accompanied by elevation in the levels of several inflammatory mediators including TNF (T. Liu et al., 1994), HMGB1 (J. B. Kim et al., 2006; J. Qiu et al., 2008), and interleukines (T. Liu et al., 1994; T. Sairanen et al., 2001). Ischemia also results in the activation of several transcription factors such as NF-κB and AP-1 (Q. Wang et al., 2007).

In this study, we used an *in vivo* model of ischemia, MCAO in mice and an *in vitro* model, OGD of primary cortical neurons to investigate a possible link between the transcription factor NF-κB and some of the inflammatory mediators reported to play a role in stroke, namely the AA cascade end product, PGE_2, together with the 3 major genes responsible for its production cPLA-2, COX-2 and mPGES-1, and TNF. Our study investigated the role of HMGB1 in ischemia using primary cortical neurons or mixed neural cultures containing neurons, astrocytes and microglia.

3.1. Neuronal IKK2 is essential for arachidonic acid cascade activation following ischemia

To investigate the activation of the AA cascade following ischemia, mice were subjected to 48 h of ischemia using MCAO. The mRNA expression of the three AA cascade genes cPLA-2, COX-2 and mPGES-1 in the ischemic core and periphery was quantified using real time RT-PCR. We observed a 298 % induction in cPLA-2 in the ischemic core and a 178 % induction in the periphery of ischemia (Figure **3.1a**). Similarly, COX-2 was up-regulated to about 212 and 207 % in the ischemic core and in the periphery of ischemia respectively (Figure 3.1b), while mPGES-1 was induced up to 293 % in the ischemic core and to lesser extent (132 %) in the periphery of ischemia (Figure 3.1.c).

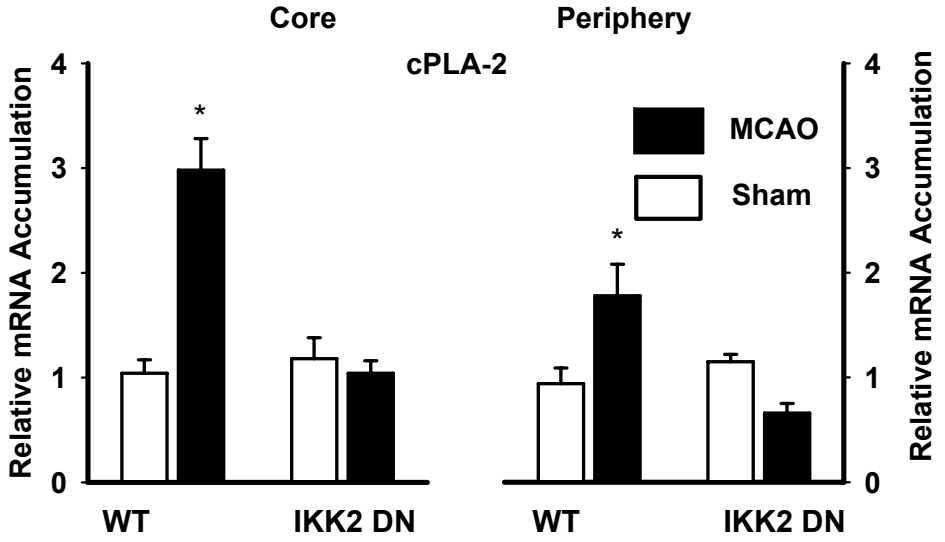
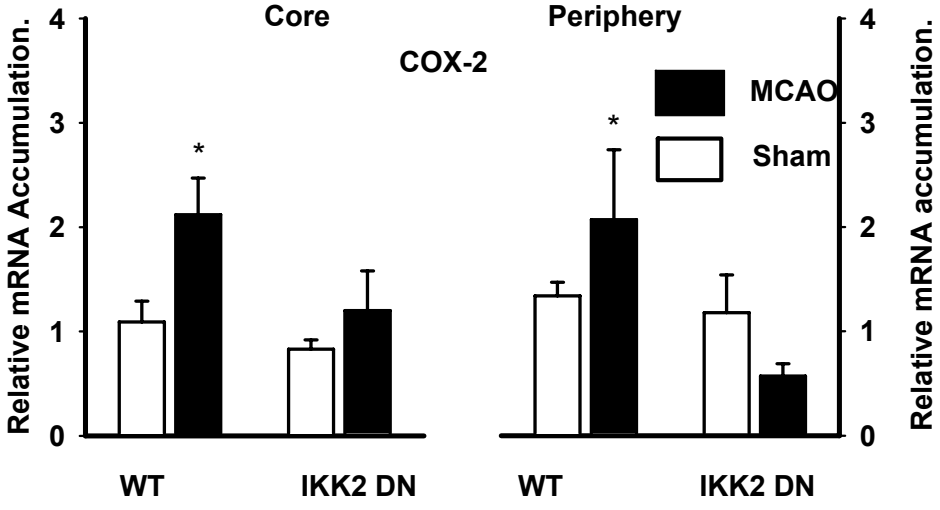
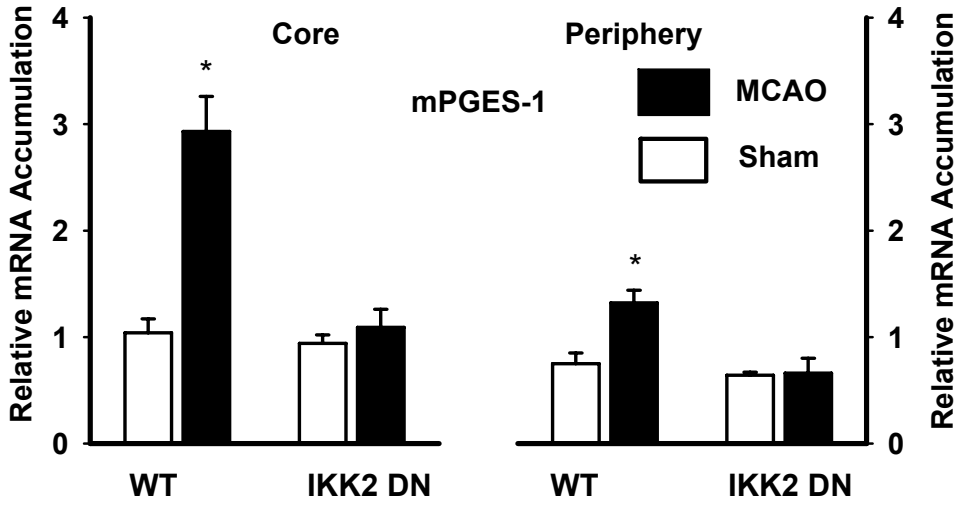


Figure 3.1: Ischemia (48 h MCAO) activates the arachidonic acid cascade,

*3.1.a) mRNA expression of cPLA-2 was increased both in core and periphery of ischemia in wild-type mice but not in mice expressing a dominant inhibitor of IKK-2 in neurons. Values are means ± SEM, n=6, * P<0.05, Student t-test.*



*3.1.b) mRNA expression of COX-2 was increased both in core and periphery of ischemia in wild-type mice but not in mice expressing a dominant inhibitor of IKK-2 in neurons. Values are means ± SEM, n=6, * P<0.05, Student t-test.*



3.1.c) mRNA expression of mPGES-1 was increased both in core and periphery of ischemia in wild-type mice but not in mice expressing a dominant inhibitor of IKK-2 in neurons. Values are means ± SEM, n=6, * P<0.05, Student t-test.

In parallel, we subjected mice expressing a dominant inhibitor of IKK-2 in neurons to cerebral ischemia (O. Herrmann et al., 2005). Fourty eight hours after MCAO, the mRNA expression of the three AA cascade genes in the ischemic core and periphery was quantified. However, we could not detect any up-regulation in the expression of cPLA-2, COX-2 and mPGES-1 in these mice after MCAO both in the ischemic core or in the periphery of ischemia (Figure 3.1a, b and c). Interestingly, these mice had less infarct volume in comparison to their littermates (O. Herrmann et al., 2005). This suggests that neuronal activation of IKK2 mediates the up-regulation of cPLA-2, COX-2 and mPGES-1 induced after ischemia. This effect may be responsible for the damage occurring in MCAO.

3.2. Oxygen glucose deprivation activates NF-κB and arachidonic acid cascade genes.

In an *in vitro* model of ischemia, primary cortical neurons were subjected to OGD for 4.5 h and allowed to recover for 24 h under normal conditions. OGD resulted in

neuronal cell death as indicated by the elevation in LDH release into the cell culture medium (Figure 3.2.a).

To test the possible involvement of NF-κB in the effects observed following OGD, we transfected primary cortical neurons with phRL-TK and pNF-κB-luc. In the latter the transcription and production of luciferase enzyme is driven by NF-κB activating signals. Neurons were transfected for 24 h before the induction of OGD (4.5 h followed by 24 h recovery). Then, the transcriptional activity of NF-κB was assessed using dual luciferase assay. Indeed, we observed a 196 % increase in the transcriptional activity of NF-κB (Figure 3.2.b), indicating the involvement of NF-κB in the effects observed in this *in vitro* model of ischemia.

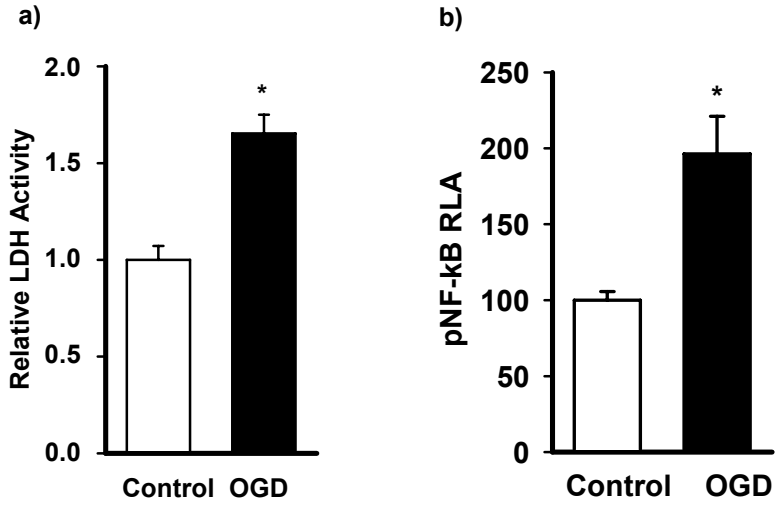
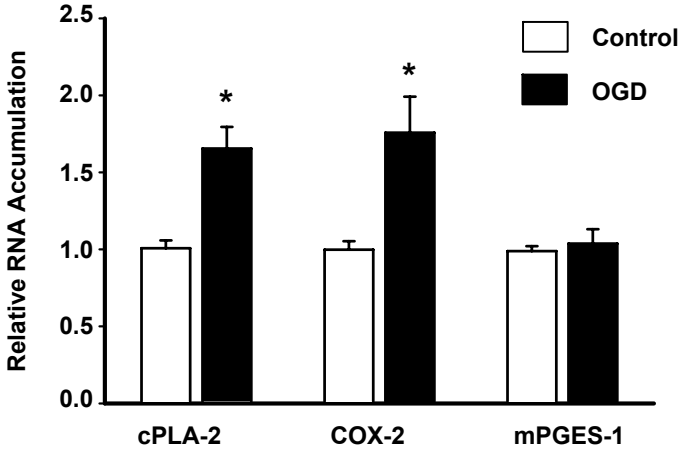


Figure 3.2: Oxygen glucose deprivation (OGD, for 4.5 h followed by 24 h recovery) of primary cortical neurons is cytotoxic and activates both NF-κB and the arachidonic acid cascade.

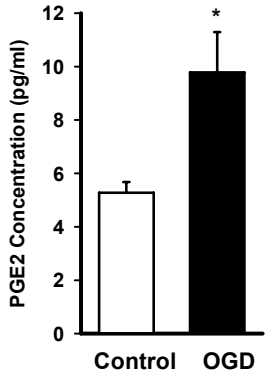
3.2.a) Primary cortical neurons release more LDH into the medium after exposure to OGD. Values are means ± SEM, n=8, * P<0.05, Student t-test.

3.2.b) The transcriptional activity of NF-κB was elevated following OGD in primary cortical neurons transfected with pNF-κB-luc. Values are means ± SEM, n=8, * P<0.05, Student t-test.

In parallel, RNA was extracted from neuronal cultures subjected to OGD to investigate the mRNA expression of the AA cascade genes by real time RT-PCR. We found an induction of cPLA-2 and COX-2 at the mRNA level (Figure 3.2.c). We also checked the release of PGE_2 in the cell culture medium after OGD using specific ELISA for PGE_2 and found the level to be elevated after OGD (Figure 3.2.d).

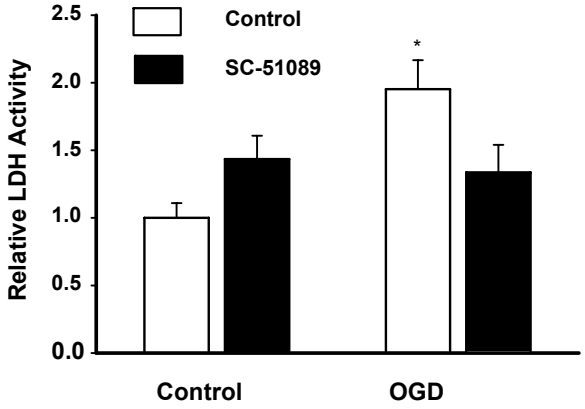


3.2.c) mRNA expression of the arachidonic acid cascade genes cPLA-2 and COX-2 in primary cortical neurons was increased following OGD. Values are means ± SEM, n=6, * P<0.05, Student t-test.



3.2.d) Primary cortical neurons release more PGE_2 into the medium after exposure to OGD. Values are means ± SEM, n=8, * P<0.05, Student t-test.

To identify the mediator responsible for the toxic effects observed after OGD, we subjected primary cortical neurons to OGD in the presence of SC-51089 (10 μM), an EP1 antagonist (P. Zhou et al., 2008) or solvent. Interestingly, SC-51089 completely abolished the toxic effects observed after OGD (Figure 3.2.e), indicating that activation of the AA cascade following ischemia and the subsequent production of PGE_2 might play a role in the toxic effects observed.



3.2.e) Blocking the EP1 receptor by the antagonist SC-51089 (10 μM) protected neurons against the toxic effects of OGD. Values are means ± SEM, n=6, * P<0.05 in comparison to non-OGD control, one way ANOVA with Tukey's post hoc test.

3.3. Tumor necrosis factor activates NF-κB and the arachidonic acid cascade genes expression.

TNF is reported to have a pro-inflammatory role after brain injuries such as ischemia (S. M. Allan and N. J. Rothwell, 2001). To investigate the effect of TNF on the transcriptional activity of NF-κB, we transfected primary cortical neurons with pNF-κB-luc vector. The cultures were transfected for 24 h, stimulated with TNF (10 ng/ml) for 6 h and then changes in the transcriptional activity were measured by means of the luciferase assay. Interestingly, TNF induced a 305 % increase in the transcriptional activity of NF-κB indicating the involvement of NF-κB in the effects produced by TNF (Figure 3.3.a).

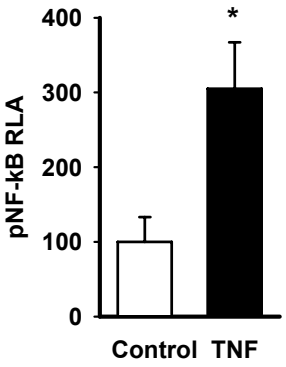
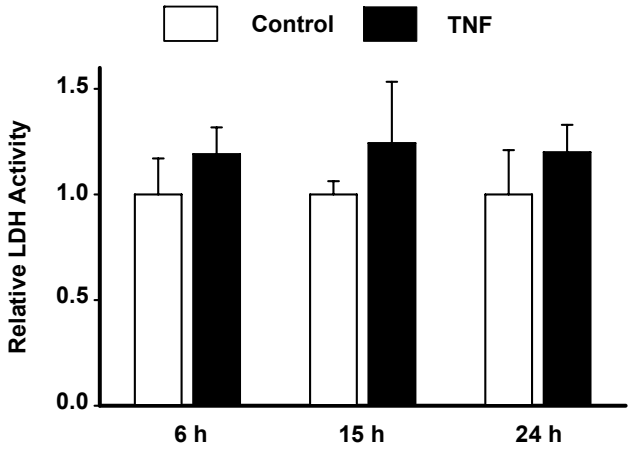


Figure 3.3:Tumor necrosis factor (TNF, 10 ng/ml) activates NF-κB and the arachidonic acid cascade genes in primary cortical neurons but does not induce cytotoxicity.

3.3.a) The transcriptional activity of NF-κB was elevated in primary cortical neurons transfected with pNF-κB-luc following stimulation with TNF for 6 h. Values are means ± SEM, n=6, * P<0.05, Student t-test.

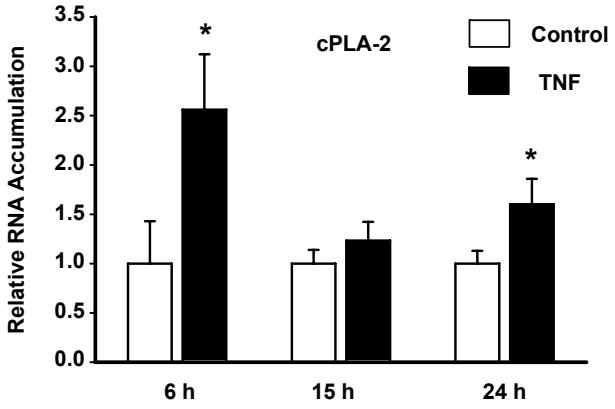
The release of LDH into the medium after TNF treatment was not changed after 6, 15 and 24 h (Figure 3.3.b).



3.3.b) TNF did not change the level of LDH released into the cell culture medium by primary cortical neurons after 6, 15 and 24 hours. Values are means ± SEM, n=6.

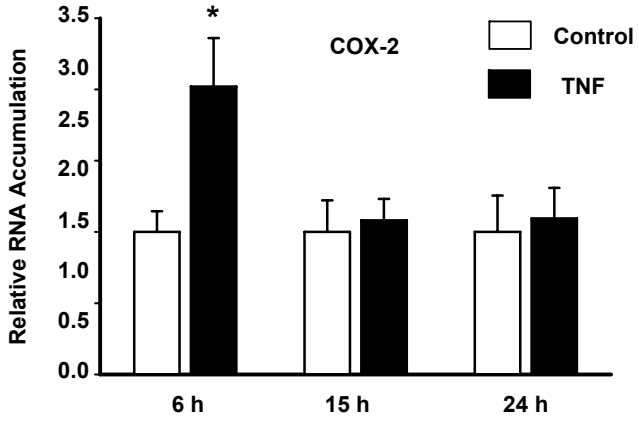
Next, we stimulated primary cortical neuronal cultures with TNF (10 ng/ml) for 6, 15 or 24 h and quantified the mRNA expression of cPLA-2, COX-2 and mPGES-1

by real-time RT-PCR. Stimulation of neurons with TNF resulted in a 256 % induction of cPLA-2 after 6 hr and a smaller elevation at later time points (Figure 3.3.c).

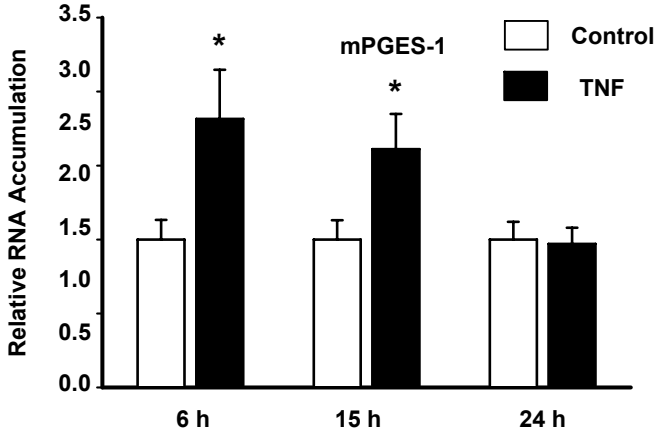


3.3.c) mRNA expression of cPLA-2 in primary cortical neurons was increased following stimulation with TNF. Values are means ± SEM, n=6, * P<0.05, Student t-test.

Similarly, COX-2 mRNA levels were elevated to 202 % after 6 hr of stimulation with TNF (Figure 3.3.d) and also mPGES-1 levels were upregulated after 6 hr and declined at 15 and 24 h (Figure 3.3.e).

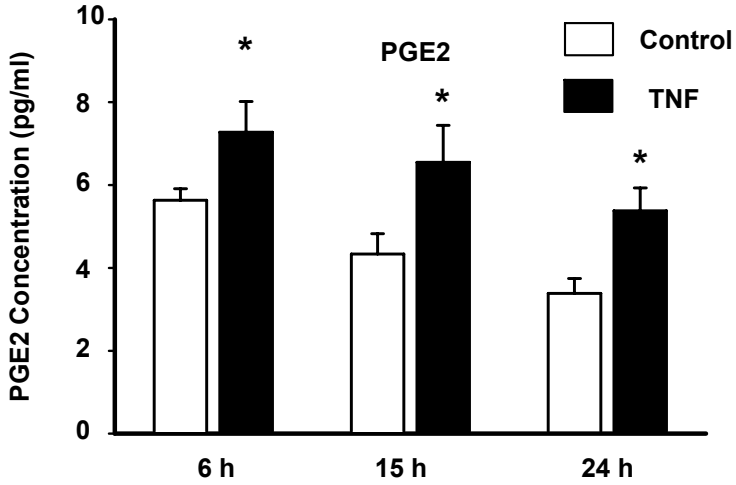


3.3.d) mRNA expression of COX-2 in primary cortical neurons was increased following stimulation with TNF. Values are means ± SEM, n=6, * P<0.05, Student t-test.



3.3.e) mRNA expression of mPGES-1 in primary cortical neurons was increased following stimulation with TNF. Values are means ± SEM, n=6, * P<0.05, Student t-test.

In parallel, we measured the levels of PGE_2 in the cell culture supernatant at the different time points following stimulation of neurons with TNF using an ELISA specific for PGE_2 and found the levels to be elevated at all time points (Figure 3.3.f).



3.3.f) Primary cortical neurons released more PGE_2 into the medium after 6, 15 and 24 h of exposure to TNF .Values are means ± SEM, n=6, * P<0.05, Student t-test.

3.4. TNF activation of NF-κB enhances the transcriptional activity of the arachidonic acid cascade genes

Next, we wanted to confirm the transcriptional regulation of the three AA cascade genes by NF-κB. To do so we used an online software (www.gene-regulation.com) and analysed the promoter region of the cPLA-2, COX-2 and mPGES-1 genes for probable NF-κB binding sites. This analysis showed several binding sites for NF-κB in the promoter regions of these genes (Figure 3.4.a).

- for cPLA-2, a single binding sequence was found at
  – 1637 (GGAATTCCCT).

- for COX-2, several binding sequences were detected at:
  - 427 (GGGGATTCCC),
  - 428 (AGGGGATTCC),
  - 591 8GGGTAGTTCC),
  - 704 (GGAAAATACC),
  - 1514 (GAAATTTCCC) and
  - 1515 (TGAAATTTCC).

- for mPGES-1, a single binding sequences was detected at
  - 801 (GGAAGGGCCA).

We constructed reporter fusion genes by inserting a short (-900/+80 bp) or a long (-1750/+80 bp) segment of the promoter sequence of the cPLA-2 and COX-2 genes into the promoterless vector pXP2. In these constructs, the transcriptional activity in the promoter region of these genes will drive the expression of luciferase. To test changes in the transcriptional activity induced in mPGES-1, we used the previously reported vectors (H. Naraba et al., 2002), which contain a short (-930/+33 bp) or a long (-1814/+33 bp) segment of the promoter region of mPGES-1 in the promoterless vector pGL3-basic to drive the expression of luciferase.

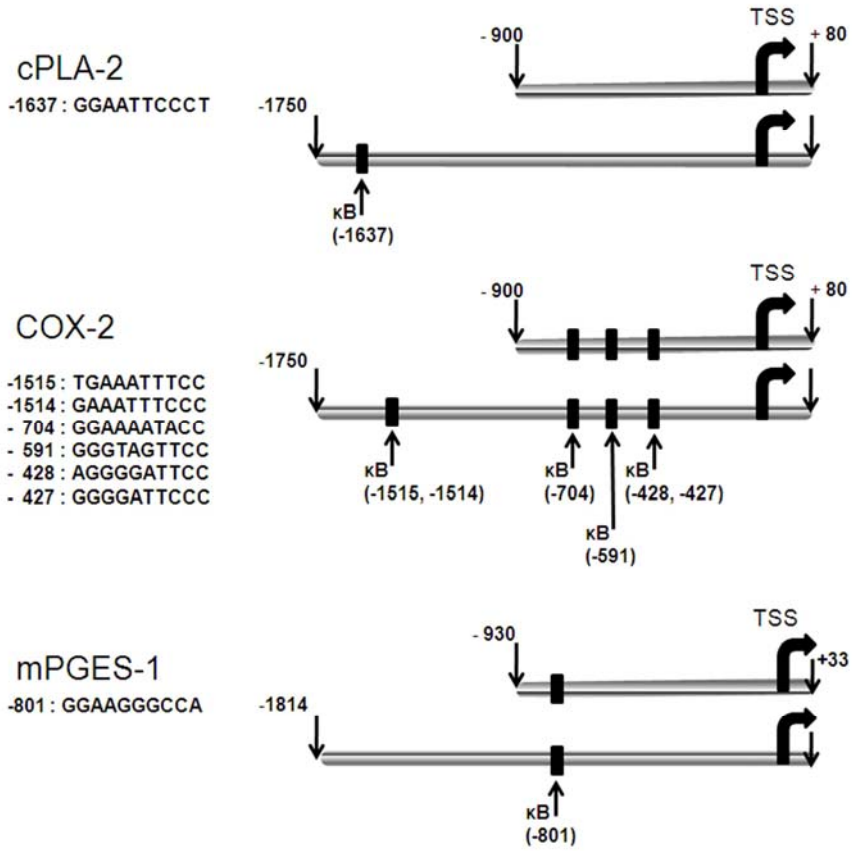
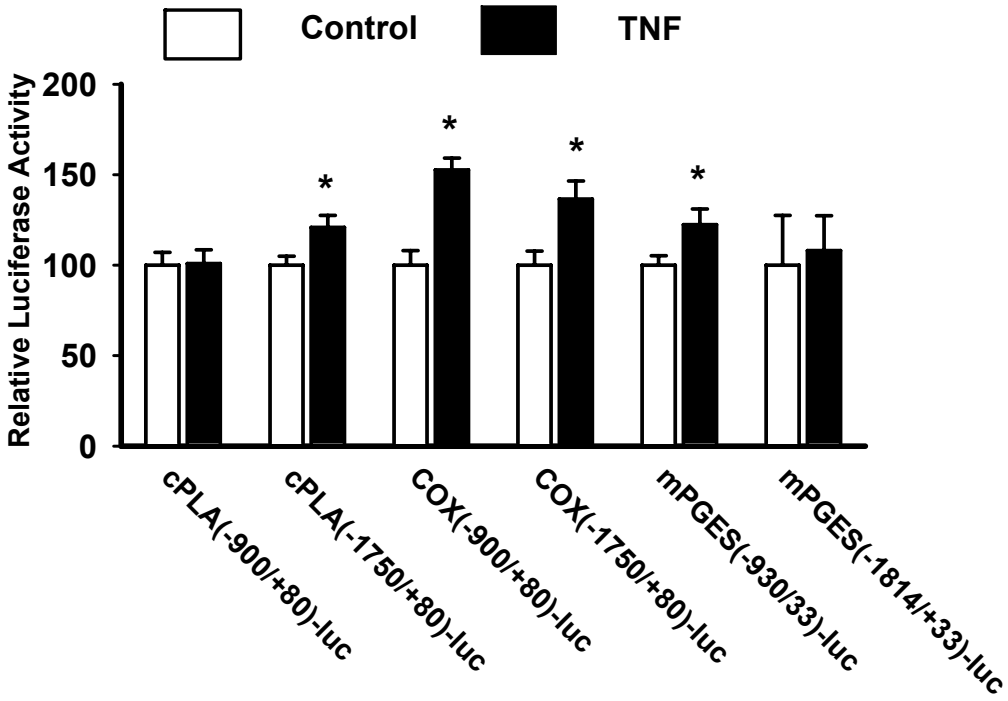


Figure 3.4. NF-κB can bind to and activate the arachidonic acid cascade genes, cPLA-2, COX-2 and mPGES-1.

3.4.a. Probable binding sites of NF-κB to the promoter regions of cPLA-2, COX-2 and mPGES-1. Binding sequences are shown in the left panel.

Primary cortical neurons were transfected with each of these constructs or the empty vector (which served as a background control) 24 h before being stimulated with TNF for 6 h. The transcriptional activity of cPLA-2 was only increased in case of the long construct cPLA-2(-1750/+80)-luc that contained the binding site for NF-κB (Figure 3.4.b). Interestingly, the transcriptional activity of COX-2 was increased in both constructs that contain NF-κB binding sites (Figure 3.4.b). However, the transcriptional activity of mPGES-1 was only increased in the case of the short construct mPGES(-930/+33)-luc (Figure 3.4.b).

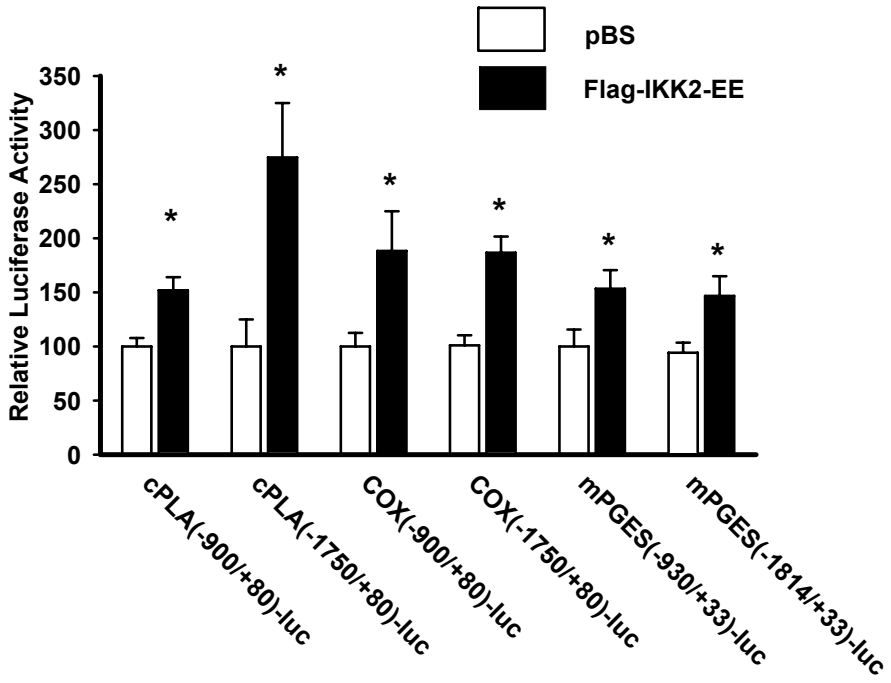


3.4.b. Stimulation by TNF (10 ng/ml for 6 h) leads to induction of the transcription of the arachidonic acid cascade genes, cPLA-2, COX-2 and mPGES-1. Values are means ± SEM, n=6, * P<0.05, Student t-test.

To further investigate the possible role of NF-κB in driving the expression of cPLA-2, COX-2 and mPGES-1, we decided to use two approaches. The first was to use an upstream activator of NF-κB, IKK2, which is responsible for the phosphorylation and degradation of the inhibitory IκB (N. D. Perkins, 2000). Once IκB is degraded, free NF-κB is immediately translocated into the nucleus where it binds to DNA inducing the expression of several genes (Q. Wang et al., 2007). The second approach was to use the active subunit of NF-κB, p65 as a direct stimulant for the transcription of cPLA-2, COX-2 and mPGES-1 in primary cortical neurons.

First, we transfected primary cortical neurons with the vector bluescript (pBS) or the constitutively active IKK2 vector (B. Baumann et al., 2007) together with phRL-TK and one of the following constructs: cPLA(-900/+80)-luc, cPLA(-1750/+80)-luc, COX(-900/+80)-luc, COX(-1750/+80)-luc, mPGES(-930/+33)-luc or mPGES (-1814/+33)-luc. Six hours after transfection, the changes in the

transcriptional activity of cPLA-2, COX-2 and mPGES-1 were evaluated by measuring the firefly luciferase activity. In parallel, the renilla luciferase activity was measured to normalize for differences in cell death or transfection efficiency. Interestingly, we found out that the transcriptional activity for both constructs of cPLA-2 was significantly elevated under the influence of the constitutively active IKK2 (Figure 3.4.c). Similarly, the transcriptional activity for COX-2 and mPGES-1 constructs was increased by the constitutively active IKK2 (Figure 3. 4. c).

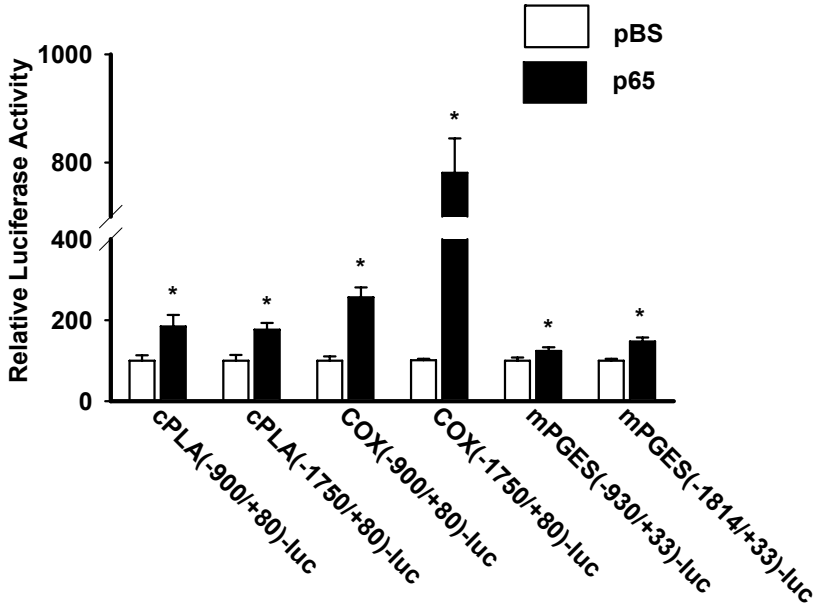


3.4.c. Increasing the activity of NF-κB by overexpressing a constitutively active IKK2 increased the transcriptional activity of the arachidonic acid cascade genes, cPLA-2, COX-2 and mPGES-1. Values are means ± SEM, n=6, * P<0.05, Student t-test.

This provides evidence that increasing the activity of NF-κB by the use of the constitutively active IKK2 construct (Flag-IKK2-EE) induces an increase in the transcription of cPLA-2, COX-2 and mPGES-1.

The second approach was to test if direct activation of NF-κB could also trigger the expression of these genes. Primary cortical neurons were transfected with phRL-TK

and an expression plasmid for p65, RcCMV-p65 (M. L. Schmitz and P. A. Baeuerle, 1991) or bluescript (pBS) together with one of the following constructs: cPLA(-900/+80)-luc, cPLA(-1750/+80)-luc, COX(-900/+80)-luc, COX(-1750/+80)-luc, mPGES(-930/+33)-luc or mPGES (-1814/+33)-luc. Six hours after transfection, the transcriptional activity of each of the genes was measured by dual luciferase assay. Differences in transfection efficiency were normalized by renilla luciferase activity. The transcriptional activity of all constructs (cPLA-2, COX-2 and mPGES-1, both the short and the long constructs) was increased by overexpression of p65 (Figure **3.4.d**).



3.4.d. Direct stimulation of NF-κB in primary cortical neurons by overexpression of p65 resulted in an elevation in the transcriptional activity of the arachidonic acid cascade genes cPLA-2, COX-2 and mPGES-1. Values are means ± SEM, n=6, * P<0.05, Student t-test.

This further confirms our conclusion that NF-κB regulates the transcription of cPLA-2, COX-2 and mPGES-1 in cultured primary cortical neurons.

3.5. Oxygen glucose deprivation-associated toxicity of primary cortical neurons is mediated through High mobility group box 1 protein release

When primary cortical neurons were subjected to OGD (4.5 hours and recovery for 24 hours), we noticed an elevation in the levels of HMGB1 in the cell culture medium (Figure 3.5.a).

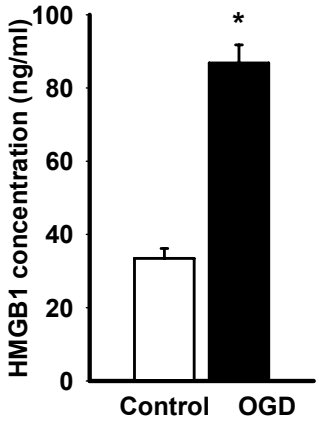
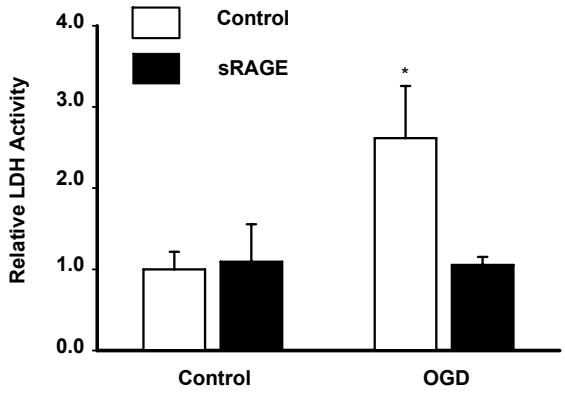


Figure 3.5: HMGB1 is associated with death of primary cortical neurons subjected to OGD (4.5 hours and 24 hours recovery).

3.5.a. Neurons release HMGB1 following OGD. Values are means ± SEM, n=8, * P<0.05, Student t-test.

HMGB1 acts on several receptors including RAGE, TLR-2 and TLR-4 and other unidentified receptors (M. T. Lotze and K. J. Tracey, 2005). To study the role of HMGB1 in the toxic effects observed after OGD, we subjected primary cortical neurons to OGD in the presence of soluble RAGE (sRAGE, 50 µg/ml), which is the decoy receptor for RAGE blocking binding of agonists to RAGE (G. Marsche et al., 2007), or solvent. Interestingly, sRAGE completely abolished the toxic effects observed after OGD (Figure 3.5.b), indicating that the release of HMGB1 following ischemia may play a role in neuronal cell death.



3.5.b. sRAGE (50 µg/ml) could protect neurons against the toxic effects of OGD. Values are means ± SEM, n=6, * P<0.05 in comparison to non-OGD control, non-OGD sRAGE and OGD sRAGE, one way ANOVA with Tukey's post hoc test.

3.6. Recombinant HMGB1 is not toxic to primary cortical neurons and does not activate NF-κB nor the expression of genes in the arachidonic acid cascade

In an attempt to confirm the role of HMGB1 in neuronal cell death following OGD, primary cortical neurons were stimulated with recombinant HMGB1 (500 ng/ml) for 24 h and cell death was investigated by measuring the release of LDH into the cell culture medium. Surprisingly, HMGB1 did not change the level of LDH released (Figure 3.6.a). To study the effects of HMGB1 on the NF-κB signaling in primary cortical neurons, we transfected primary cortical neurons with pNF-κB-luc vector for 24 hours, then stimulated with HMGB1 (500 ng/ml) for another 24 h before the transcriptional activity of NF-κB was measured by luciferase assay. The transcriptional activity of NF-κB was not different after stimulation with HMGB1 (Figure 3.6.b).

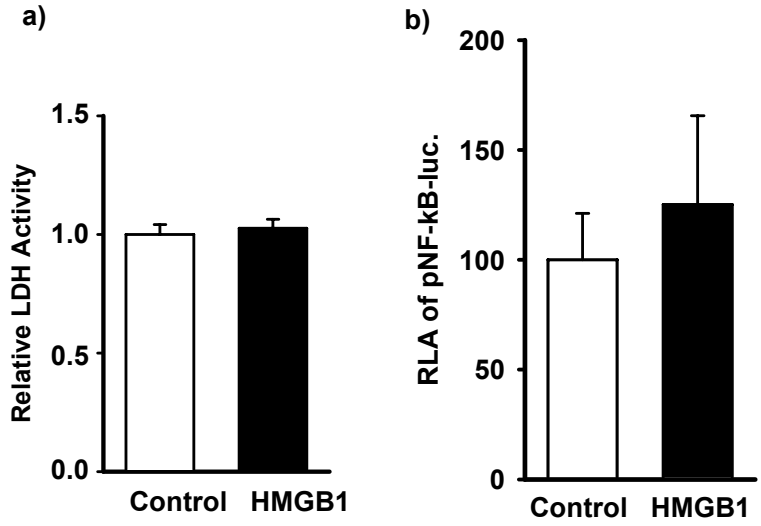


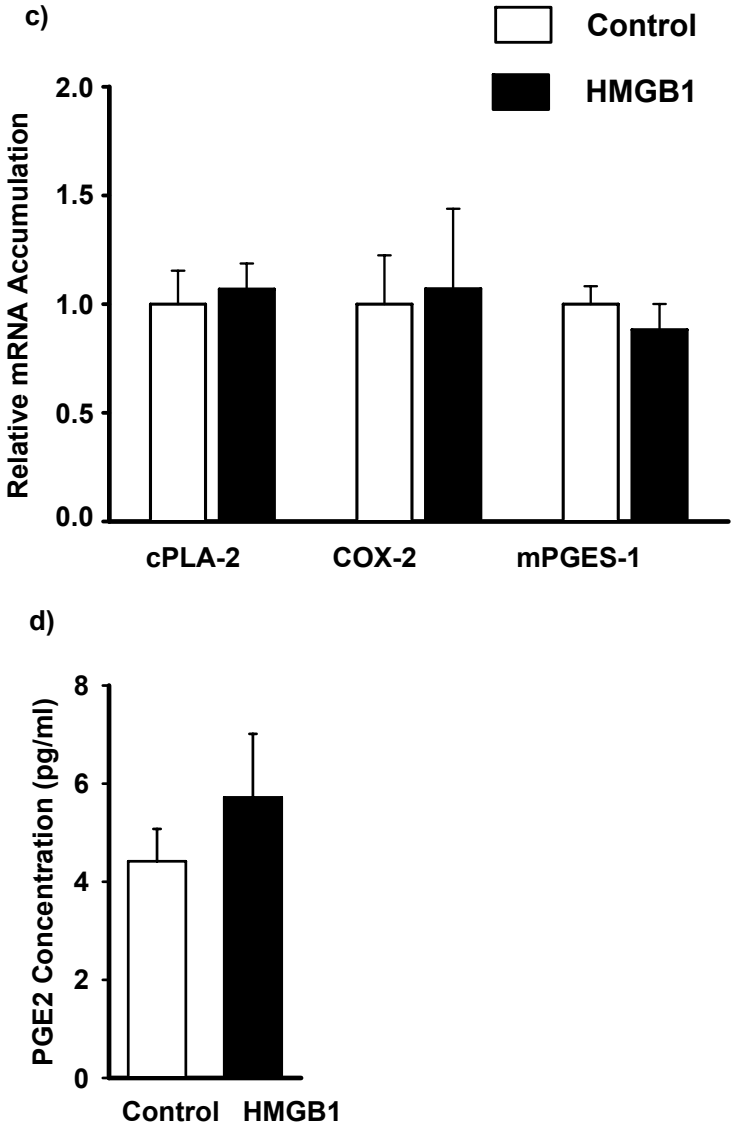
Figure 3.6. Stimulation of primary cortical neurons with the recombinant HMGB1 (500 ng/ml for 24 hours) is not toxic and does not activate NF-κB nor the arachidonic acid cascade.

3.6.a. Stimulation of primary cortical neurons with the recombinant HMGB1 does not change the amount of LDH released into the medium. Values are means ± SEM, n=8.

3.6.b. Stimulation of primary cortical neurons (transfected with pNF-κB-luc) with the recombinant HMGB1 does not change the transcriptional activity of NF-κB. Values are means ± SEM, n=6.

To identify a possible effect of HMGB1 on the AA cascade, primary cortical neurons were stimulated with HMGB1 for 24 hours. Then, RNA was isolated and used to perform real time RT-PCR for cPLA-2, COX-2 and mPGES-1. We found no change in the expression of cPLA-2, COX-2 and mPGES-1 (Figure 3.6.c).

During this experiment, cell culture medium was collected and used to measure the level of PGE_2 using a specific ELISA. However, this revealed no change in the level of PGE_2 in response to HMGB1 (Figure 3.6.d) indicating that HMGB1 is not acting through NF-κB or the AA cascade in primary cortical neurons.



3.6.c. Stimulation of primary cortical neurons with recombinant HMGB1 did not induce the expression of the arachidonic acid cascade genes (cPLA-2, COX-2 and mPGES-1. Values are means ± SEM, n=6.

3.6.d. Stimulation of primary cortical neurons with the recombinant HMGB1 did not change the level of PGE2 released into the medium. Values are means ± SEM, n=6.

3.7. Neuronal glial interaction mediates the toxic effect of HMGB1

To identify whether HMGB1 has a cell specific effect or not, we cultured pure cortical neurons, glial mixtures (astrocytes and microglia), pure astrocytes, pure microglia or mixed neural cultures containing all three types of cells (neurons, astrocytes and microglia) and stimulated each of these cultures with recombinant HMGB1 (500 ng/ml) for 24 h. Cell death was evaluated by measuring the amount of LDH released in the medium. Interestingly, HMGB1 had a toxic effect only on mixed neural cultures containing neurons, astrocytes and microglia (Figure 3.7.a), indicating the importance of the interaction between these cell types to mediate the effect(s) of HMGB1.

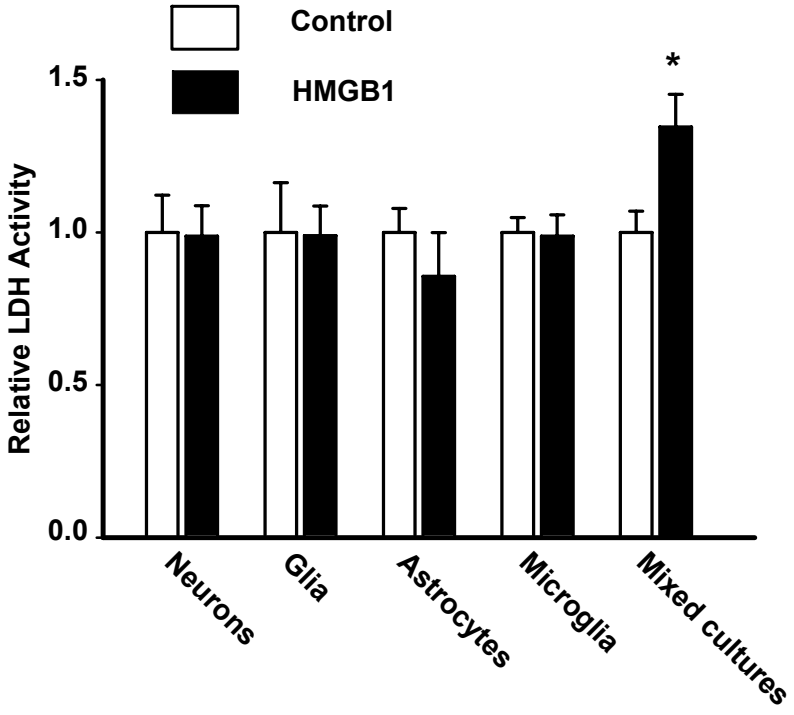
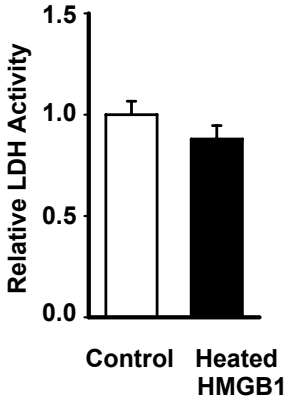


Figure 3.7. The interaction between neurons and glia is needed to mediate the effect of HMGB1.

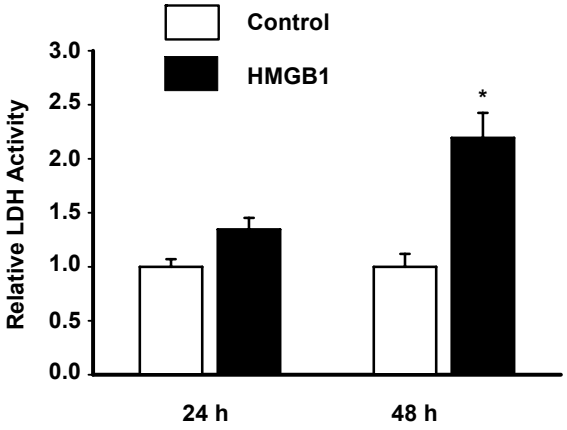
3.7.a. Recombinant HMGB1 (500 ng/ml for 24 hours) is toxic only to cultures containing neurons, microglia and astrocytes (mixed neural cultures). Values are means ± SEM, n=6, * P<0.05, Student t-test.

Since the toxic effect of HMGB1 was quite mild (only 25 – 35 % increase in LDH leakage from the cells into the cell culture medium), we wanted to confirm that this effect is really specific and not due to any possible contaminant endotoxins (although the effect was cell type specific and the levels of contaminants were far beyond the level reported to have any biological effects). For this purpose, we stimulated mixed neural cultures with heat-inactivated HMGB1 (heated at 95 °C for 5 min) and measured LDH release after 24 h. Indeed, heat-inactivated HMGB1 had lost its toxic effects and the levels of LDH in the culture medium were not significantly different from the control group (Figure 3.7.b).



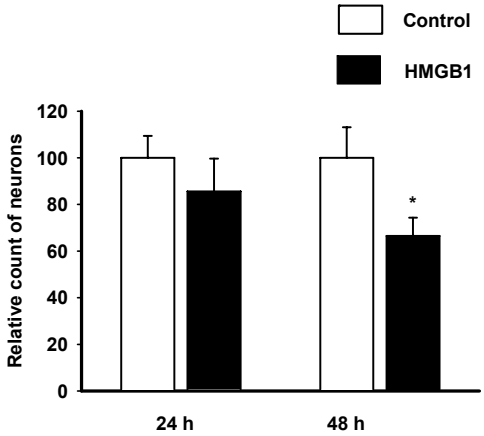
3.7.b. Heat-inactivated HMGB1 is non toxic to mixed neural cultures proving specificity of the effects observed. Values are means ± SEM, n=6.

Interestingly, when we stimulated mixed neural cultures with HMGB1 and measured changes in LDH level after 24 and 48 h, we found that mixed neural cultures release more LDH after 48 h than after 24 h in response to HMGB1 (Figure 3.7.c) confirming the specificity of the effect of HMGB1.

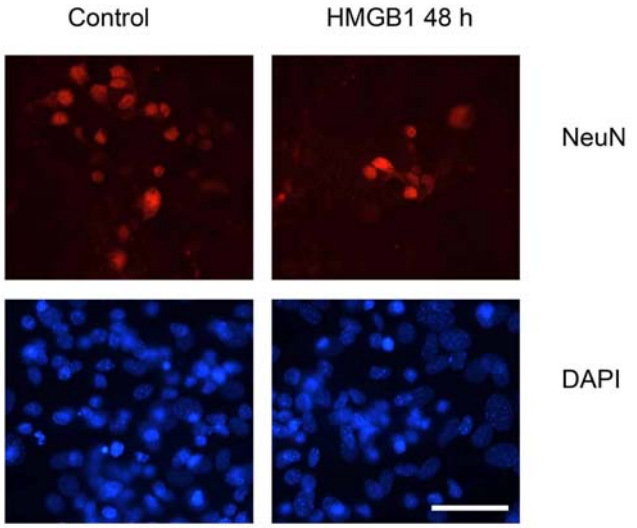


3.7.c. Time-dependant effect of HMGB1 stimulation of mixed neural cultures showing that the effect is increased by increasing the exposure time. Values are means ± SEM, n=8, * P<0.05 in comparison to 24 and 48 h control, one way ANOVA with Dunn's post hoc test.

In order to identify the mechanism of the observed effect of HMGB1, we wanted to identify the cell population which was killed in response to HMGB1. To do so we compared the number of neurons in our mixed neural cultures treated with HMGB1 or solvent by counting the number of NeuN-positive cells in these cultures. Indeed, HMGB1 reduced the number of neurons in mixed neural cultures and this was significantly different after 48 h (Figure 3.7.d and e).



3.7.d. Stimulation of mixed neural cultures with recombinant HMGB1 reduced the number of neurons in these cultures. Values are means ± SEM, n=8, * P<0.05 in comparison to 24 h control, one way ANOVA with Dunn's post hoc test.



3.7.e. IHC of NeuN in mixed neural cultures showing the reduction in the count of NeuN-positive cells 48 hours after stimulation with HMGB1. Scale bar 50 µm.

3.8. RAGE on microglia mediates the toxic effect of HMGB1 on mixed neural cultures

In order to define the molecular basis for the effect of HMGB1 on mixed neural cultures, we wanted to investigate the presence of HMGB1 receptors in our cell culture model. To do so we performed RT-PCR for TLR-2, TLR-4 and RAGE using cDNA obtained from whole brain, pure neuronal cultures, or pure microglial cultures and spleen tissue as a positive control. We used the levels of GAPDH as a house keeping gene. Whole brain expressed all receptors and both TLR-2 and TLR-4 were expressed by microglia, while neurons expressed only TLR-2 and RAGE (Figure 3.8.a).

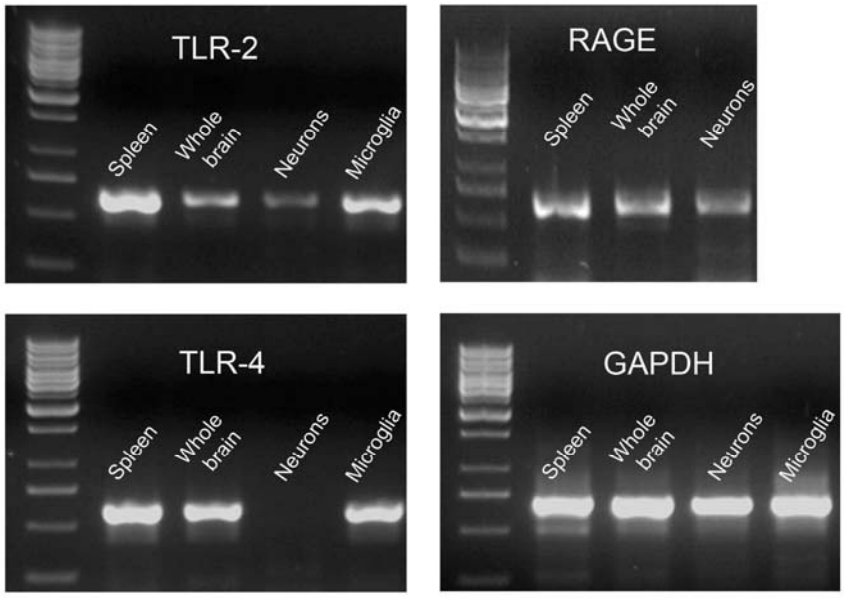
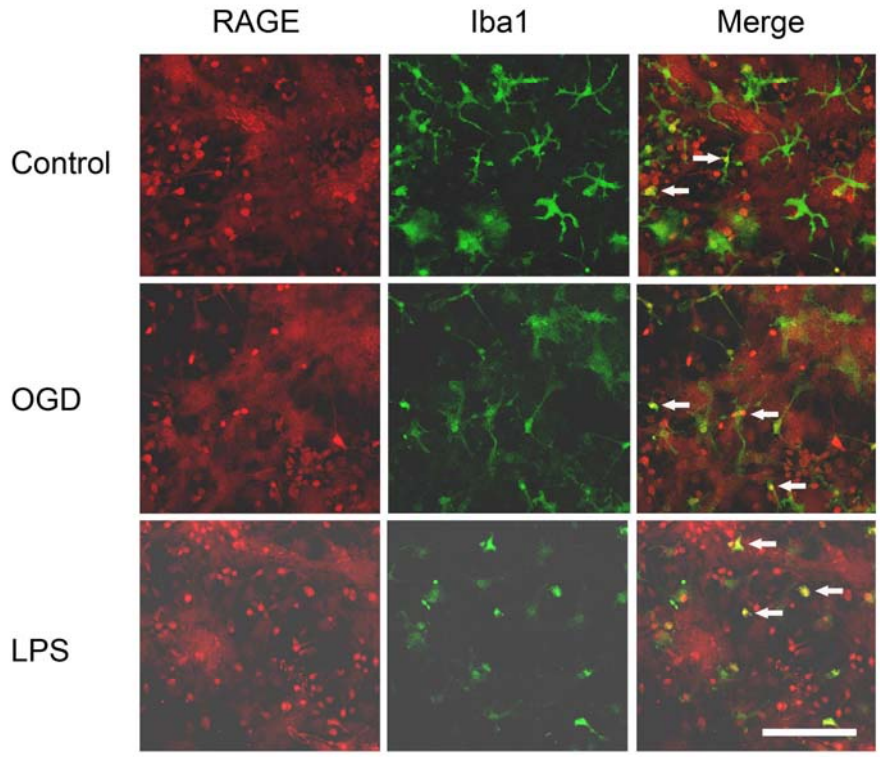


Figure 3.8. RAGE on microglia mediates the toxic effects of HMGB1.

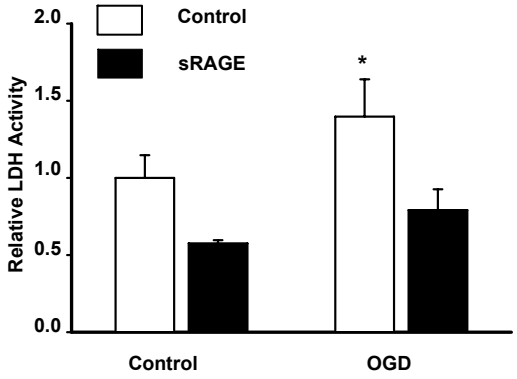
3.8.a. RT-PCR confirmed the presence of HMGB1 receptors in our cell culture model.

To investigate the expression of RAGE by microglia, we stained glial cultures for Iba-1, which is a marker for microglia, together with RAGE and found that some microglia express RAGE (Figure 3.8.b). Interestingly, stimulation of microglia with LPS (1 µg/ml) for 24 h or OGD resulted in an increase in the number of microglia expressing RAGE (Figure 3.8.b) which was accompanied by a change in the morphology of microglia from the resting ramified shape to the stimulated amoeboid shape (Z. Xiang et al., 2006).



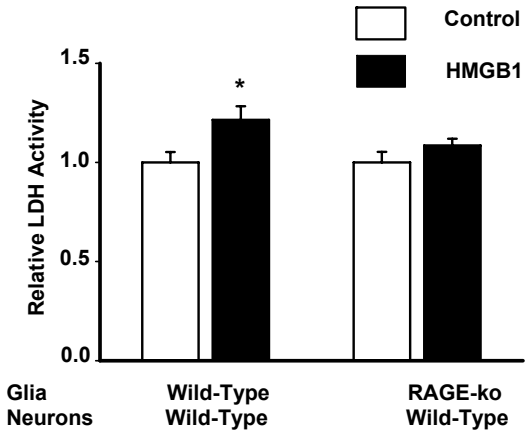
3.8.b. IHC of Iba-1 and RAGE showing the colocalization of both in some microglia and this colocalization is increased after stimulation with OGD or LPS (1 µg/ml for 24 hours). Scale bar 50 µm.

To confirm the role of RAGE in neuronal cell death following ischemia, we subjected mixed neural cultures to OGD in presence of sRAGE (50 µg/ml) or solvent and investigated cell death by comparing the amount of LDH released in the cell culture medium between the two treatment groups. sRAGE, indeed, protected mixed neural cultures against the toxic effects of OGD (Figure 3.8.c).



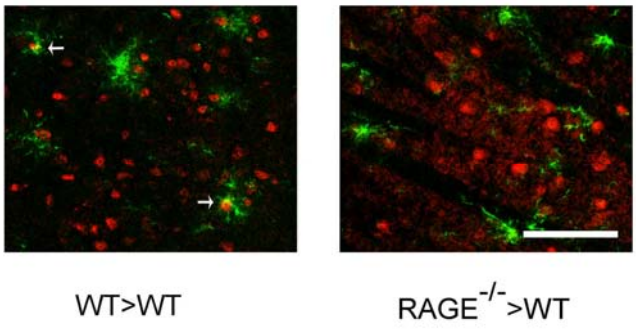
3.8.c. sRAGE (50 µg/ml) protected mixed neural cultures against OGD induced cell death. Values are means ± SEM, n=8, * P<0.05 in comparison to Non-OGD and OGD sRAGE, one way ANOVA with Dunn's post hoc test.

To further confirm the role of RAGE in mediating the effects of HMGB1 in our mixed neural culture model, we prepared mixed neural culture from different genotypes. Cultures containing RAGEko glia with wild-type neurons or cultures containing wild-type glia and wild-type neurons were stimulated with HMGB1. Interestingly, absence of RAGE on glia abolished the toxic effect of HMGB1 on mixed neural cultures (Figure 3.8.d) which implies an important role of glial RAGE in mediating the effects of HMGB1.



3.8.d. Absence of RAGE on glia abolished the toxic effect of HMGB1 on mixed neural cultures. Values are means ± SEM, n=8, * P<0.05, Student t-test.

To confirm the effect of RAGE *in vivo*, bone marrow from wild-type or RAGEko mice was transplanted into irradiated wild-type mice. Six weeks later, MCAO was performed for 48 hours which showed that mice receiving RAGEko bone marrow had smaller infarcts (S. Muhammad et al., 2008). In order to find the role of RAGE on migrating macrophages we stained sections from mice of both groups for RAGE and Iba-1 and found less colocalization between RAGE and Iba-1 in mice which received bone marrow from RAGEko mice indicating that absence of RAGE on migrating macrophages could protect the mice against the damage occurring after MCAO (Figure 3.8.e).



3.8.e. IHC of sections obtained 48 hours after MCAO in mice transplanted with wild-type or RAGEko bone marrow showing less colocalization between RAGE and Iba-1 on migrating macrophages in case of bone marrow derived from RAGEko mice. Scale bar 50 µM.

3.9. Microglia/macrophage mediates the toxic effect of HMGB1 on mixed neural cultures.

In order to test the role of microglia in the observed response to HMGB1, we wanted to deplete mixed neural cultures from microglia and investigate the response of microglia-depleted cultures to HMGB1. To do so we applied two approaches:

First, we treated wild-type glial cultures with liposomes containing PBS or clodronate (25 % W/V) which is reported to deplete microglia and macrophages *in vivo* (N. van Rooijen and E. van Kesteren-Hendrikx, 2002). Treatment of glial

cultures with liposomes (0.2 ml / 3 ml medium) for 24 hours reduced the number of Iba-1-positive cells (Figure 3.9.a and b).

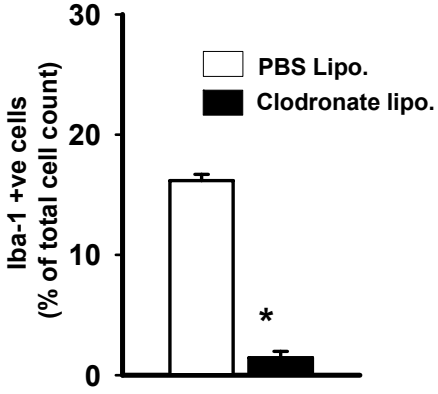
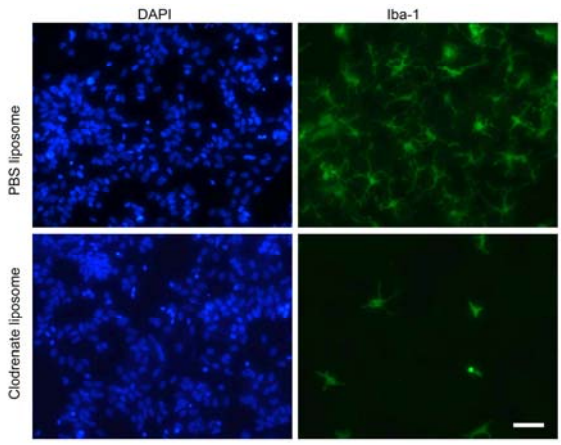


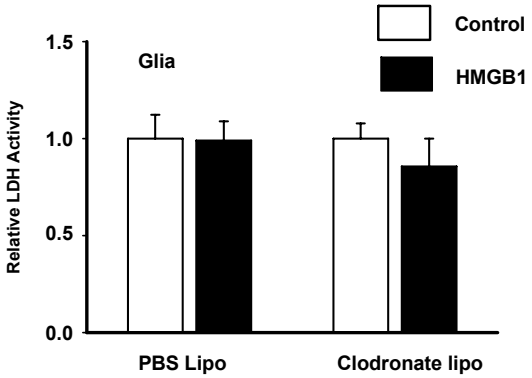
Figure 3.9. Microglia and macrophages mediate the toxic effect of HMGB1 on mixed neural cultures.

3.9.a. Treatment of glial cultures with clodronate containing liposomes reduced the number of Iba-1-positive cells. Values are means ± SEM, n=4, * P<0.05, Student t-test.



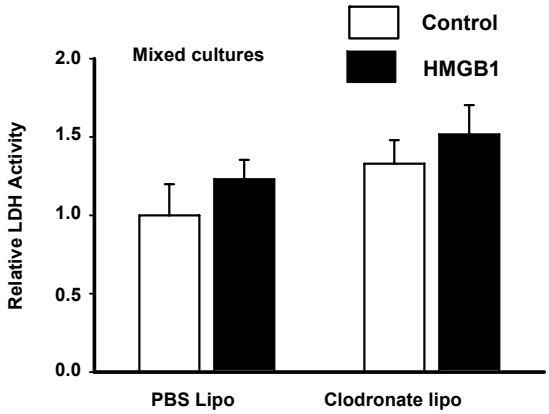
3.9.b. IHC of Iba-1 in glial cultures after treatment with PBS or clodronate containing liposomes showing the depletion of microglia by clodronate treatment. Scale bar 50 µM.

After treatment with liposomes, HMGB1 did not increase the level of LDH in the cell culture medium (Figure 3.9.c).



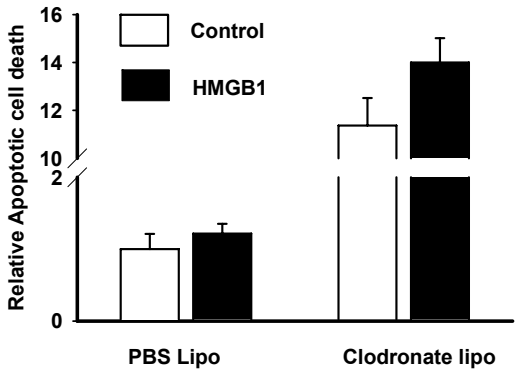
3.9.c. Stimulation with HMGB1 of glial cultures pretreated with PBS or clodronate containing liposomes showed no significant difference in cell death between the different groups. Values are means ± SEM, n=6.

When neurons were cocultured with glia pretreated with PBS or clodronate containing liposomes HMGB1 had no effect on liposome-treated cultures (PBS or clodronate) (Figure 3.9.d). However, the basal level of LDH release was higher in case of cultures pretreated with clodronate containing liposomes, indicating that treatment of cultures with liposome had affected all cell populations in the culture and lead to loss of the effect of HMGB1 in both cases.



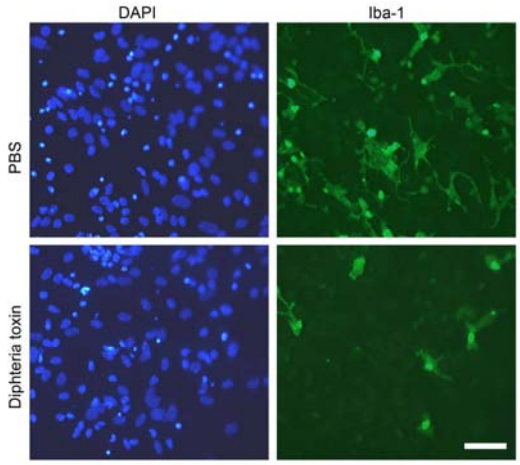
3.9.d. Stimulation with HMGB1 (500 ng/ml) of mixed neural cultures in which glia were pretreated with PBS or clodronate containing liposomes did not affect LDH release. Values are means ± SEM, n=6.

When we checked cell death by apoptosis using a specific ELISA kit for DNA histone complexes, we found that HMGB1 caused a mild but non-significant increase in cell death by apoptosis in both groups. However, the basal level was much higher in the group that was pretreated with clodronate containing liposomes (Figure 3.9.e) indicating that at least in our cell culture model, liposome treatment affects more cells than only microglia.



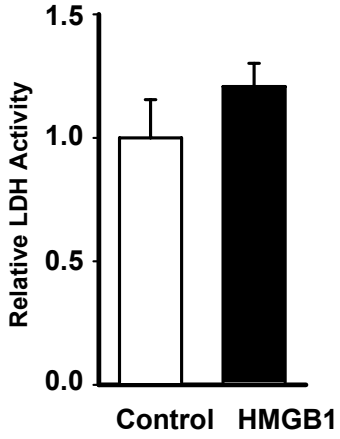
3.9.e. HMGB1 did not cause a significant increase in apoptotic cell death after pretreatment with PBS or clodronate containing liposomes. Values are means ± SEM, n=6.

The second approach to deplete mixed neural cultures from microglia was to prepare glia from CD11b-DTR mice which express diphtheria toxin receptor only in microglia (V. Stoneman et al., 2007). Treatment of cultures prepared from these mice with diphtheria toxin should only kill microglia. Indeed, treatment with diphtheria toxin (100 ng/ml) for 24 hours resulted in a reduction in the number of Iba-1-positive cells (Figure 3.9. f).



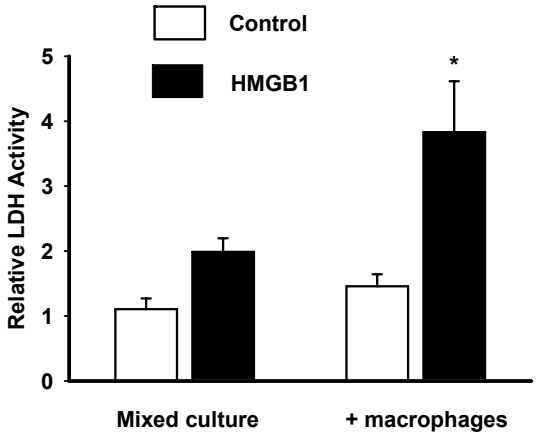
3.9.f. IHC of Iba-1 in glial cultures prepared from CD11b-DTR-positive mice after treatment with diphtheria toxin (100 ng/ml) or solvent showing the depletion of microglia in these cultures. Scale bar 50 µM.

Stimulation with HMGB1 (500 ng/ml for 24 hours) caused only a mild nonsignificant increase in LDH release (Figure **3.9.g**), indicating that the presence of microglia is indeed important to mediate the effect of HMGB1.



3.9.g. Stimulation with HMGB1 of mixed neural cultures in which CD11b-DTR-positive glia were pretreated with diphtheria toxin or solvent showed a mild nonsignificant elevation in LDH release. Values are means ± SEM, n=6.

As an alternative approach, we decided to mimic the *in vivo* situation following ischemia, which is characterized by an increase in the migration of blood derived immune cells such as macrophages (R. Tanaka et al., 2003), neutrophils (J. M. Hallenbeck, 1996) and other cell types to the site of the injury. We cocultured mixed neural cultures with peritoneal macrophages. When mixed neural cultures grown with macrophages were stimulated with HMGB1 for 48 hours we noticed a large increase in cell death from 198 % to 383 % (Figure 3.9.h).



3.9.h. Coculture of mixed neural cultures with peritoneal macrophages increased the toxic effect of HMGB1. Values are means ± SEM, n=6, * P<0.05 in comparison to non-HMGB1 controls, one way ANOVA with Tukey's post hoc.

3.10. Prostaglandin E_2 released from microglia mediates the toxic effect of HMGB1

In an attempt to identify the mediator responsible for the interaction between the different cell populations in our cell culture model, we cultured pure microglia and stimulated them with HMGB1 for 24 h before cell culture medium was removed and processed for the measurement of LDH and PGE_2 using a specific ELISA. We found that HMGB1 could increase the levels of PGE_2 released in the medium by about 30 % (Figure 3.10.a). This was not accompanied by any change in the cell

death as seen from the similar level of LDH released into the cell culture medium in both groups (Fig 3.10.b)

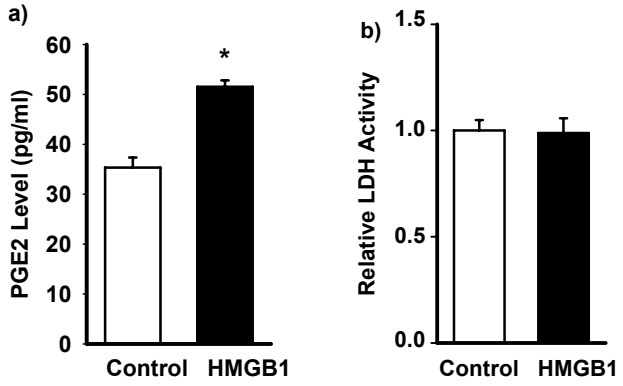
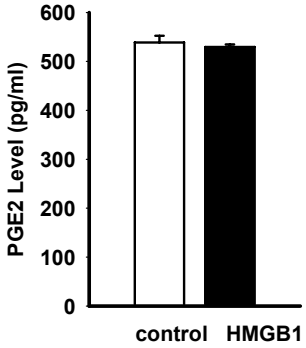


Figure 3.10. HMGB1 (500 ng/ml for 24 hours) induced the release of PGE2 from microglia and causes cell death in mixed neural cultures.

3.10.a). Stimulation with HMGB1 of pure microglial cultures resulted in an elevation in the release of PGE2 into the medium. Values are means ± SEM, n=8, * P<0.05, Student t-test.

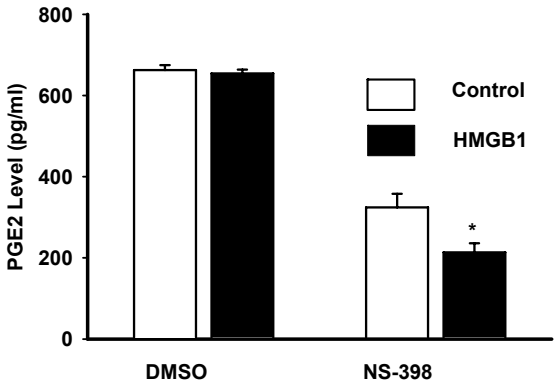
3.10.b). Stimulation with HMGB1 of pure microglial cultures did not cause any change in the release of LDH into the medium. Values are means ± SEM, n=8.

Nevertheless, we could not find similar changes in PGE_2 in case of mixed neural cultures stimulated with HMGB1 for 24 h (Figure 3.10.c).



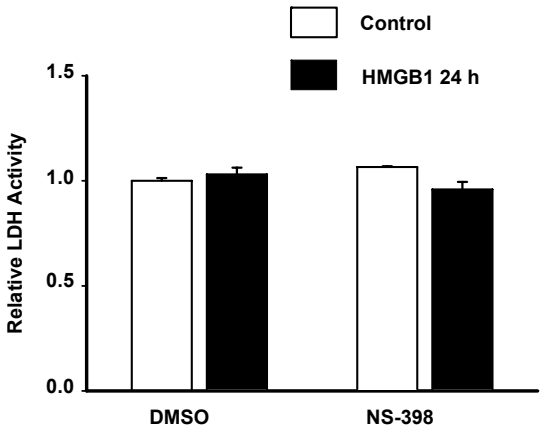
3.10.c. Stimulation with HMGB1 of mixed neural cultures did not change the level of PGE_2 released in the medium. Values are means ± SEM, n=8.

To confirm a possible role of PGE_2 released by microglia in mediating the effect of HMGB1 on mixed neural cultures, we applied NS-398, a well established inhibitor of COX-2 activity (H. C. Choi et al., 2008). NS-398 (10 µM) was applied to mixed neural cultures 1 hour before stimulation with HMGB1 for 24 h. Then PGE_2 levels in the cell culture medium were quantified in comparison to a control group, which received only the solvent DMSO. Indeed, pretreatment with NS-398 reduced the level of PGE_2 in the medium to less than 50 % of the solvent treated groups (Figure 3.10.d).



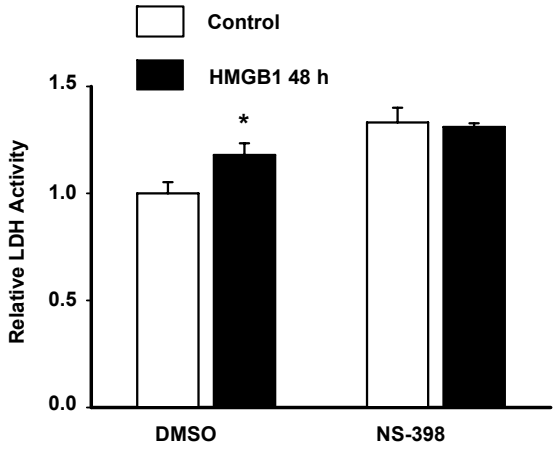
3.10.d) Stimulation with HMGB1 of mixed neural cultures pretreated with NS-398 (10 µM) or solvent resulted in a significant reduction in the level of PGE2 released into the medium. Values are means ± SEM, n=8, * P<0.05 in comparison to DMSO treated groups, one way ANOVA with Dunn's post hoc test.

At the same time, media were also used to measure the levels of LDH as a marker of cell death. Surprisingly, after 24 h with NS-398 or DMSO, the toxic effect of HMGB1 was not detectable anymore and the level of LDH released into the cell culture medium was the same between all groups (Figure 3.10.e).



3.10.e. Stimulation with HMGB1 (for 24 hours) of mixed neural cultures pretreated with NS-398 (10 µM) or DMSO showed no change in the level of LDH released into the medium. Values are means ± SEM, n=6

However, when the stimulation time was increased to 48 h, HMGB1 had a toxic effect on DMSO treated mixed neural cultures but not in NS-398 treated cultures (Figure 3.10.f) although the effect after treatment with DMSO was less than that observed without DMSO (Figure 3.10.f) thus, blocking PGE_2 production can rescue mixed neural cultures from the toxic effects of HMGB1.



3.10.f. Stimulation with HMGB1 (for 48 hours) of mixed neural cultures pretreated with NS-398 (10 µM) or DMSO showed that blocking the release of PGE2 by NS-398 protected the cultures against the toxic effects of HMGB1. Values are means ± SEM, n=6, * P< 0.05, Student t-test.

3.11. Ischemia induces the release of HMGB1 from neurons but not from glia

In order to identify the cell population which releases HMGB1 after OGD, glial cultures were subjected to OGD. Then, we stained the cells for HMGB1 together with GFAP as a marker for astrocytes or CD11b as a marker for microglia and compared the colocalization under normal and OGD conditions. Colocalization was not changed by OGD indicating that in our model of OGD, astrocytes and microglia retain their content of HMGB1 following OGD (Figure 3.11.a).

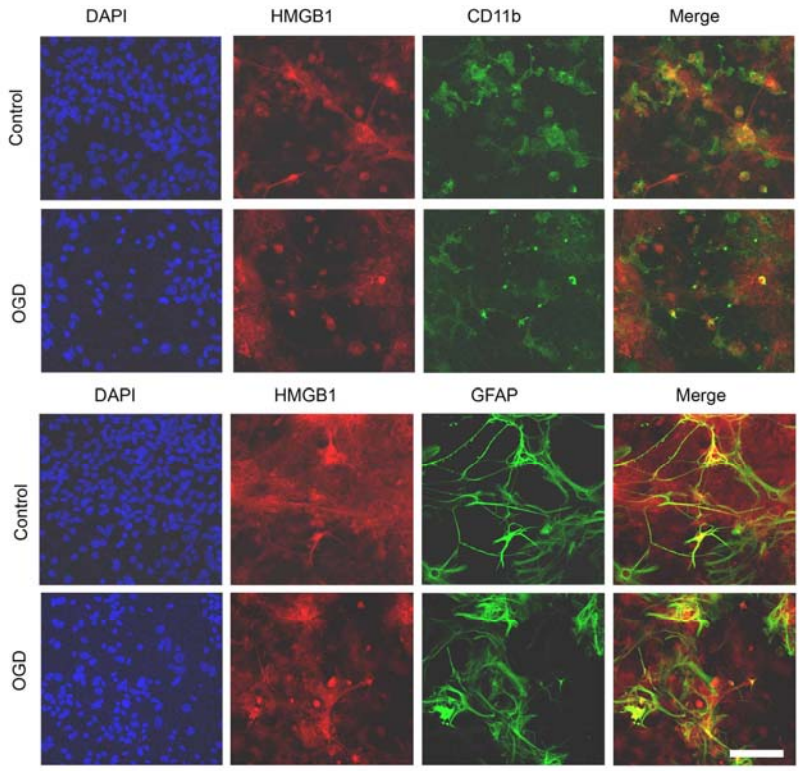
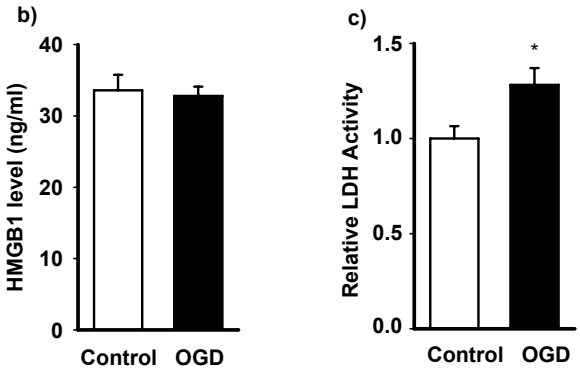


Figure 3.11. OGD (4.5 hours and 24 hours recovery) of mixed neural cultures induced the release of HMGB1 from neurons not microglia or astrocytes.

3.11.a. IHC of glial cultures after OGD showing the colocalization of HMGB1 and the microglial marker CD11b or the astrocytic marker GFAP under normal and OGD conditions. Scale bar 50 μM.

We also used media obtained from glial cultures subjected to OGD to measure the levels of HMGB1 released into the medium and found no change between control

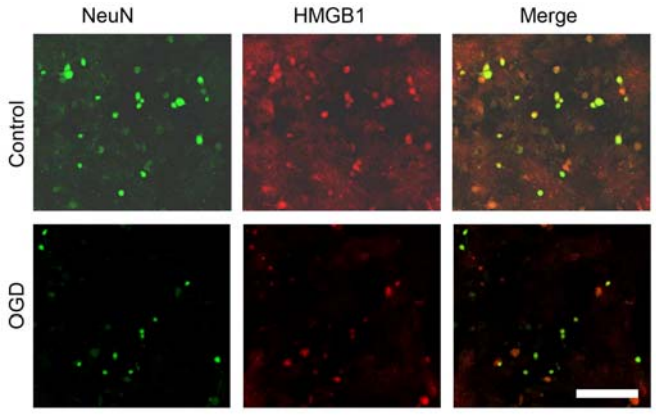
and OGD groups (Figure 3.11.b), although there was a mild increase in LDH release after OGD (Figure 3.11.c).



3.11.b. OGD of glial cultures did not increase the release of HMGB1 into the cell culture medium. Values are means ± SEM, n=6.

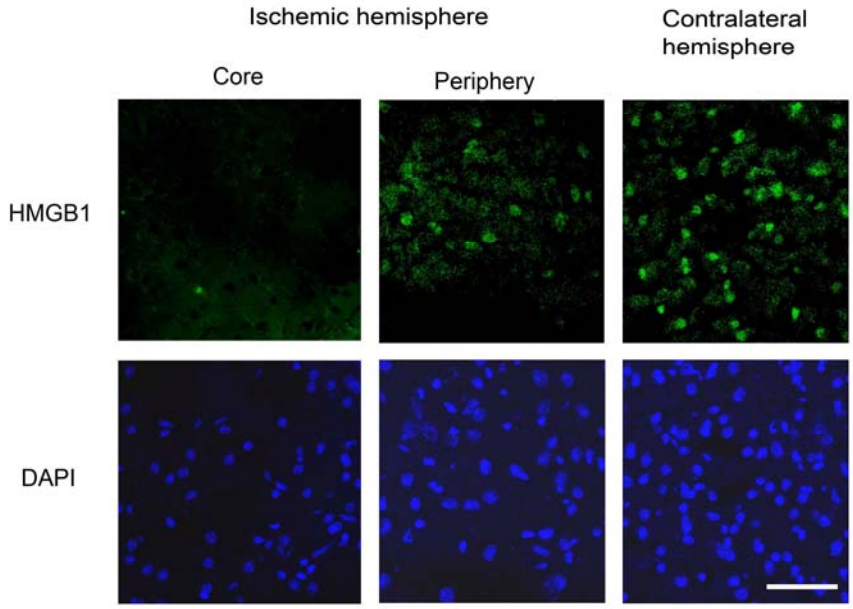
3.11.c. OGD of glial cultures caused an increase the release of LDH into the cell culture medium. Values are means ± SEM, n=6, * P< 0.05, Student t-test.

When mixed neural cultures were subjected to OGD and then stained for HMGB1 and NeuN as a marker of neurons, we found a decrease in the number of double positive cells and also a reduction in the HMGB1 staining intensity in NeuN-positive cells (Figure 3.11.d), indicating that OGD induced the release of HMGB1 from neurons.



3.11.d. IHC of mixed neural cultures after OGD showing less colocalization of HMGB1 and the neuronal marker NeuN after OGD. Scale bar 50 µM.

To confirm the observation that HMGB1 is released after ischemia, sections obtained from mice subjected to MCAO for 4 hours were stained for HMGB1. Interestingly, there was a profound reduction in the number of cells, which express HMGB1 in the ischemic hemisphere (periphery) and a complete loss in the core of ischemia in comparison to the contralateral hemisphere (Figure 3.11.e) indicating that mild ischemic insults as OGD (*in vitro*) or in the periphery of ischemia (*in vivo*) caused the release of HMGB1 from some cells (probably neurons), while, severe ischemic insults as in the core of ischemia (*in vivo*) caused the release of HMGB1 from all cells.



3.11.e. IHC of sections obtained 4 hours after MCAO showing the complete loss of HMGB1 signal in the core of ischemia and the partial loss in the periphery of ischemia. For comparison, nuclei were stained by DAPI. Scale bar 50 µM.

4. Discussion

4.1. Summary of the results

In the present study, we show that ischemia induces genes involved in the AA cascade both *in vivo* and *in vitro* and that the induction is dependent on NF-κB signaling. The *in vitro* experiments also showed that PGE_2 was associated with the neurotoxicity observed after OGD of primary cortical neurons. We also show that the three genes of the AA cascade, cPLA-2, COX-2 and mPGES-1 have probable binding sites where NF-κB can bind and induce their expression.

Stimulation of primary cortical neurons with TNF activated NF-κB and induced the expression of the three genes, cPLA-2, and COX-2 and mPGES-1 both at the transcriptional level and at the mRNA level. TNF also induced the production of the AA cascade end product PGE_2. In addition, increasing the activity of NF-κB by the use of the constitutively active IKK-2 construct or the active subunit p65 both resulted in a significant induction in the transcription of the three target genes.

In addition, we studied the effect of the late mediator of inflammation HMGB1 following ischemia and confirmed its role in mediating some of the toxic effects observed after ischemia. HMGB1 was released from neurons (but not from microglia or astrocytes) after ischemia. Blocking its effects using the decoy receptor sRAGE was found to be protective both *in vivo* and *in vitro.* However, stimulation of primary cortical neurons with recombinant HMGB1 was not toxic and toxicity was only induced in cultures containing neurons, astrocytes and microglia (mixed neural cultures).

The toxic effect of HMGB1 was dependant on the presence of RAGE on glia (microglia in mixed neural cultures and microglia or infiltrating macrophages *in vivo* after MCAO). Moreover, the toxicity following OGD was abolished by sRAGE both *in vivo* and *in vitro.* Depletion of microglia *in vitro* showed that microglia are important for mediating the toxic effect of HMGB1 and also the application of macrophages on the cultures exacerbated the toxicity induced by HMGB1. We

found that microglia release PGE_2 in response to HMGB1 and blocking the production of PGE_2 protected against the toxic effects of HMGB1.

4.2. Mechanisms of stroke associated damage

Stroke (focal cerebral ischemia) affects 15 million people worldwide each year and is a leading cause of death or long-term disability (J. H. Yi et al., 2007). However, the only drug approved for clinical use in most countries is the thrombolytic agent recombinant tissue plasminogen activator (rt-PA) which is only given to 4–5% of patients due to the short therapeutic time window of 3 h in which thrombolysis is safe (A. R. Green, 2008). Therefore, new treatments are needed that are still effective when administered with some delay after onset of stroke.

Stroke in experimental animals and humans leads to an infarct with a core where the blood flow is decreased by up to 100 % surrounded by a penumbra with about 90 % reduction in blood flow (J. H. Yi et al., 2007). Following stroke, oxygen and glucose insufficiency in the core lead to mitochondrial failure and inability of the cells to maintain ionic gradients across the membrane resulting in necrotic cell death.

Anoxic depolarization in the core is accompanied by elevated extracellular potassium and glutamate, intracellular calcium, generation of free radicals, oxidative stress and lactic acidosis which adds to the overall neuronal damage. However, in the penumbra, ischemia itself is not sufficiently severe to cause cell death and penumbral neurons could be rescued by therapies given within the first 6 hours following stroke (P. Lipton, 1999).

Neuroinflammation is a host defense mechanism which aimed to neutralize the insult and restore the normal structure and function of brain. All neural cells, including microglia, astrocytes, neurons, and oligodendrocytes, participate in inflammatory responses. However, microglia play the most important role. Microglial cells are activated during a CNS injury and initiate a rapid response that involves cell migration, proliferation and release of cytokines/chemokines, which stimulate

phospholipases A2 and cyclooxygenases. This results in breakdown of membrane glycerophospholipids with the release of AA and DHA (A. A. Farooqui et al., 2007).

AA and its metabolites play an important role in inflammation (D. Tassoni et al., 2008). Oxidation of AA produces pro-inflammatory prostaglandins, leukotrienes, and thromboxanes, while, DHA is metabolized to resolvins and neuroprotectins, that inhibit the generation of prostaglandins, leukotrienes, and thromboxanes (A. A. Farooqui et al., 2007). All isoforms of PLA2 and COX in the brain were reported to be stimulated after inflammation and this involved the NF-κB-mediated induction of TNF-a, IL-1β, and chemokines (A. A. Farooqui et al., 2007).

4.3. Stroke induces the arachidonic acid cascade genes through NF-κB activation

Following MCAO, we observed an upregulation in the mRNA expression of the three AA cascade genes cPLA-2, COX-2 and mPGES-1 both in the ischemic core and its periphery. Interestingly, when mice expressing a dominant inhibitor of IKK-2 in neurons were subjected to MCAO, we could not detect similar elevations in the expression of cPLA-2, COX-2 and mPGES-1 indicating that, MCAO can induce the expression of the three AA cascade genes cPLA-2, COX-2 and mPGES-1 and that the expression of the AA cascade genes depends on intact NF-κB signaling. Recently it was shown that transient ischemia resulted in an induction of cPLA-2 (C. Nito et al., 2008), COX-2 (E. Candelario-Jalil and B. L. Fiebich, 2008) and mPGES-1 (Y. Ikeda-Matsuo et al., 2006).

A link between NF-κB and the AA cascade genes was previously reported although in different models and cell types. Blocking NF-κB was shown to reduce the expression and release of phospholipases (M. Lappas et al., 2004), and COX-2 was previously reported to be a target of NF-κB (B. Kaltschmidt et al., 2002). In addition, inhibition of NF-κB signaling through blockade of IKK2 was recently shown to decrease the expression of COX-2, mPGES-1 and the production of PGE_2 in LPS-activated rat microglia (A. C. de Oliveira et al., 2008). However, in this study we

provide evidence that NF-κB activity in neurons is essential for the induction of the AA cascade genes after MCAO and OGD.

4.4. OGD induces the arachidonic acid cascade genes and NF-κB

In addition, OGD of primary cortical neurons, an *in vitro* model of ischemia, (F. He et al., 2008), was accompanied by an induction in the transcriptional activity of NF-κB. Activation of NF-κB following ischemia was previously described both *in vivo* (A. Schneider et al., 1999) and *in vitro* (A. Cardenas et al., 2000). OGD was also accompanied by an increase in the expression of the AA cascade genes cPLA-2 and COX-2 and by an increase in the production of PGE_2 in accordance with previous experiments (E. Candelario-Jalil et al., 2003; C. Yokota et al., 2004).

Blocking EP1 receptor (using the blocker SC-51089) resulted in protection of the cultures against the toxic effects observed after OGD providing further evidence for the toxic effect of PGE_2. Our in vitro findings confirm the work of Kawano et al that EP1 is the main PGE_2 receptor mediating neurotoxicity (T. Kawano et al., 2006). However, they used a different insult.

Ischemia is known to induce the activity of several transcription factors including NF-κB which controls the expression of several genes involved in inflammatory conditions such as ischemia. Cerebral ischemia also induces the production of several cytokines such as TNF, IL1β and leads to the release of HMGB1 which is a late mediator of inflammation known to be released passively or actively at late stages following inflammatory conditions such as sepsis, heat shock and ischemia.

The activation of NF-κB is well known to contribute to the injury following ischemia (A. Schneider et al., 1999). NF-κB is present in an inactive state, complexed to IκB proteins in the cytoplasm. However, upon stimulation, IκB proteins are phosphorylated, ubiquitinated and degraded by proteasome releasing free NF-κB to translocate to the nucleus (M. Schwaninger et al., 2006). This is mediated mainly by the IKK complex composed of IKK1 (or IKKα), IKK2 (or IKK β) and IKK3 (or IKK γ).

4.5. TNF induces the arachidonic acid cascade genes and NF-κB

Ischemia is known to increase the levels of TNF (L. Zhou et al., 2008). Therefore, we decided to use TNF as a stimulant of primary cortical neurons to study the mechanism(s) of the toxic effect observed after ischemia. In the tested concentration of TNF we observed no neurotoxicity but found activation of the transcriptional activity of NF-κB in accordance with previous results (G. Bonizzi and M. Karin, 2004).

Also we found an induction in the mRNA expression of the three AA cascade genes cPLA-2, COX-2 and mPGES-1 in primary cortical neurons stimulated with TNF at different time point. This was also accompanied by an increase in the production of PGE_2. TNF was known to activate cPLA-2 and thus increase the release of AA which is then metabolized to produce eicosanoids which are potent inflammatory mediators (M. Kronke and S. Adam-Klages, 2002). The expression of COX-2 was reported to be induced by TNF (Y. C. Chang et al., 2003) and also mPGES-1 expression could be induced by TNF (K. Subbaramaiah et al., 2004).

In order to find a possible link between the induction in the three AA cascade genes and the activation of NF-κB transcriptional activity that was induced in primary cortical neurons stimulated with TNF, we created reporter fusion genes in which the transcriptional activity of each of the AA cascade genes cPLA-2, COX-2 and mPGES-1 can be induced by binding of NF-κB to their prompter regions. We found that stimulation of neurons transfected with these reporter fusion genes with TNF induced the transcription of cPLA-2, COX-2 and mPGES-1 indicating that induction of NF-κB activity by TNF can trigger the transcription of the three AA cascade genes. In addition, we found that increasing the activity of NF-κB using the constitutively active IKK2 construct or p65 (active subunit of NF-κB) also resulted in an increase in the transcription of the three AA cascade genes which clearly indicated that, NF-κB regulates the expression of the three AA cascade genes, cPLA-2, COX-2 and mPGES-1. Hence the production of PGE_2 which mediates some of the toxic effects observed after ischemia is controlled by NF-κB.

4.6. HMGB1 is associated with ischemia induced neuronal cell death

In our *in vitro* model of ischemia (OGD) of primary cortical neurons we found an increase in the level of HMGB1 released into the medium which was also reported by others following ischemia (R. S. Goldstein et al., 2006). However, when glial cultures were subjected to OGD we found no change in the levels of HMGB1 released into the medium indicating that, our mild model of ischemia can induce the release of HMGB1 from neurons but not from glia. The effects of HMGB1 are mediated mainly through its interaction with RAGE. TLR2 and TLR4 are other membrane receptors that bind HMGB1 in addition to RAGE (J. S. Park et al., 2004; J. S. Park et al., 2006), although a recent study has questioned this finding (J. Tian et al., 2007).

HMGB1 is a nonhistone DNA binding protein that is widely expressed in various tissues including the brain and was thought to be important only for the stabilization of nucleosomal structures and the facilitation of gene transcription (M. Bustin, 1999). However, recently, it was shown to act as a cytokine-like mediator of delayed endotoxin lethality and acute lung injury (H. Wang et al., 1999).

HMGB1 can be secreted actively from macrophages and monocytes or passively released by necrotic cells (J. B. Kim et al., 2006). It has been also shown that HMGB1 is released in the brain, after cytokine stimulation and after ischemia (J. Qiu et al., 2008). However, actively released HMGB1 is highly acetylated by nuclear acetyltransferase before secretion, whereas passively released HMGB1 is not acetylated (T. Bonaldi et al., 2003) and so acetylated HMGB1 may have different biological properties and functions that differ from passively released or recombinant HMGB1.

Extracellular HMGB1 may exert its effects through different receptors including RAGE, TLR-2 and TLR-4 (J. R. Klune et al., 2008). Absence of RAGE (L. G. Bucciarelli et al., 2008) or TLR-2 (S. C. Tang et al., 2007) or TLR-4 (U. Kilic et al., 2008) is protective to animals subjected to ischemia.

RAGE is a multi-ligand membrane receptor, which can be activated by several ligands in cerebral ischemia. Hyperglycemia, a common finding in stroke patients (J. F. Scott et al., 1999), enhances the production of AGEs that have been shown to contribute to neurotoxicity during ischemic stroke (G. A. Zimmerman et al., 1995). In addition, there is evidence that Aβ is involved in the pathogenesis of ischemic brain injury (M. Koistinaho and J. Koistinaho, 2005) and that S100 proteins are released during ischemia and modulate its outcome (D. Kogel et al., 2004). However, the most obvious RAGE activator in stroke seems to be HMGB1 that is known to be released from necrotic cells (B. Degryse et al., 2001; P. Scaffidi et al., 2002).

4.7. HMGB1 is not acting through NF-κB nor the arachidonic acid cascade in primary cortical neurons

To test whether HMGB1 mediates some of the toxic effects of OGD, we treated primary cortical neuronal cultures with the decoy receptor sRAGE before and during OGD and found that sRAGE abolished the toxic effects observed after ischemia indicating a role of HMGB1 in that toxic effect. Protective effects were also observed by blocking RAGE in other models of ischemic injury (S. Zeng et al., 2004). However, stimulation of primary cortical neurons with the recombinant HMGB1 did not induce cell death (no change in the amount of LDH released into the medium), did not increase the transcriptional activity of NF-κB, did not induce the mRNA expression of the three AA cascade genes (cPLA-2, COX-2 and mPGES-1) and did not increase the production of PGE_2 indicating that in our model of primary cortical neurons, recombinant HMGB1 is not acting through NF-κB or the AA cascade.

4.8. Neuronal glial interaction mediates the toxic effects of HMGB1

We wondered if the effects of endogenous HMGB1 released passively after OGD differ from that of the recombinant HMGB1 or if the effect was cell specific. To answer this question, we applied recombinant HMGB1 on different primary cells (pure cortical neurons, mixed glia, pure microglia, primary astrocytes or mixed neural cultures) and found that HMGB1 is toxic only to cultures which contained

neurons, astrocytes and microglia (mixed neural cultures) indicating that interaction between these cells is required to mediate the toxic effects of recombinant HMGB1.

4.9. HMGB1 is toxic to neurons in mixed neural culture

In order to determine the mechanism of that interaction, we tried to solve several questions. First, we wanted to identify the cells which were dying in response to HMGB1 and found out that neurons were killed after exposure of mixed neural cultures to HMGB1 and this was more evident after prolonged exposure time.

Second, was to identify the receptor responsible for this effect, the first candidate being RAGE, we treated mixed neural cultures with sRAGE before and during OGD and found that the cultures were protected against the toxic effects of OGD by treatment with sRAGE, which is similar to the effect seen in pure cortical neurons and with previous reports (S. Zeng et al., 2004).

4.10. RAGE on glia is essential for the neurotoxic effect of HMGB1

The third aim was to identify the cells which express RAGE and are responsible for mediating the toxic effect of recombinant HMGB1. RAGE is localized on all types of brain cells. Activation of RAGE on neurons has been shown to stimulate the production of reactive oxygen species and to lead to the death of neuron-like cell lines (S. D. Yan et al., 1996; A. M. Vincent et al., 2006). RAGE is involved in the recruitment of neutrophils by HMGB1 (V. V. Orlova et al., 2007) and neutrophil recruitment contributes to ischemic brain injury, at least in transient cerebral ischemia (R. L. Zhang et al., 1995), suggesting that the cells mediating the RAGE effect could be neutrophils.

However, when we stimulated mixed neural cultures, in which glia lack RAGE, with recombinant HMGB1, they were not affected in contrast to cultures in which glia express RAGE indicating that the presence of RAGE on glia is essential for the toxic effects of HMGB1 on mixed neural cultures. This was also confirmed by bone marrow transplantation of RAGEko or wild-type marrow into irradiated wild-type mice followed after 6 weeks by MCAO. We found that mice receiving RAGEko

marrow had smaller infarcts and their brains contained fewer RAGE expressing macrophages confirming that absence of RAGE on infiltrating macrophages can reduce the damage occurring after MCAO.

To further identify the individual glial cell type responsible for mediating the toxic effect of HMGB1 *in vitro*, we depleted mixed neural cultures from microglia using clodronate liposomes in wild-type cultures or diphtheria toxin in cultures prepared from CD11b-DTR mice and found a tendency towards a reduced toxic effect of HMGB1 suggesting that mainly microglia mediate the toxic effect of HMGB1. To further confirm the role of microglia, we added peritoneal macrophages to mixed neural cultures before stimulating them with recombinant HMGB1 and found that the presence of macrophages exacerbates the response of mixed neural cultures to recombinant HMGB1.

Since macrophages enhanced the toxic effect of HMGB1 on the viability of mixed neural cultures, we propose that RAGE activated by HMGB1 functions as a sensor of necrotic cell death at the core of the ischemia and mediates the activation of brain macrophages, mainly immigrant macrophages.

4.11. HMGB1 induces the release of PGE_2 from microglia which has a neurotoxic effect

The next aim was to identify the mediator responsible for the interaction between microglia and neurons after HMGB1 stimulation. We observed an increase in the level of PGE_2 released into the medium after stimulation of microglia by HMGB1 and found that blocking the production of PGE_2, with the COX-2 inhibitor NS-398 protected mixed neural cultures from the toxic effects of recombinant HMGB1 indicating that PGE_2 might be responsible for the interaction between neurons and microglia that mediated the toxic effects of HMGB1.

4.12. Suggested model for the interaction between neurons and microglia after ischemia

In conclusion, we propose that PGE_2 released by neurons and/or microglia mediate some of the toxic effects of ischemia. Ischemia (among other effects) activates NF-κB in neurons that leads to the induction of genes involved in PGE_2 synthesis which mediates a neurotoxic effect by acting on EP1 receptors. In addition to that, ischemia induces neuronal death and the necrotic neurons release HMGB1 acting on RAGE receptor on microglia/macrophages. RAGE activation stimulates the AA cascade in microglia/macrophages and the subsequent production of PGE_2. PGE_2 released by microglia/macrophages act on neurons producing a kind of feedback loop which increases the neurotoxic effect occurring after ischemia (Figure 4.1). a similar model was proposed by Block et al., 2007 but they did not investigate the role of HMGB1 released from neurons (M. L. Block et al., 2007).

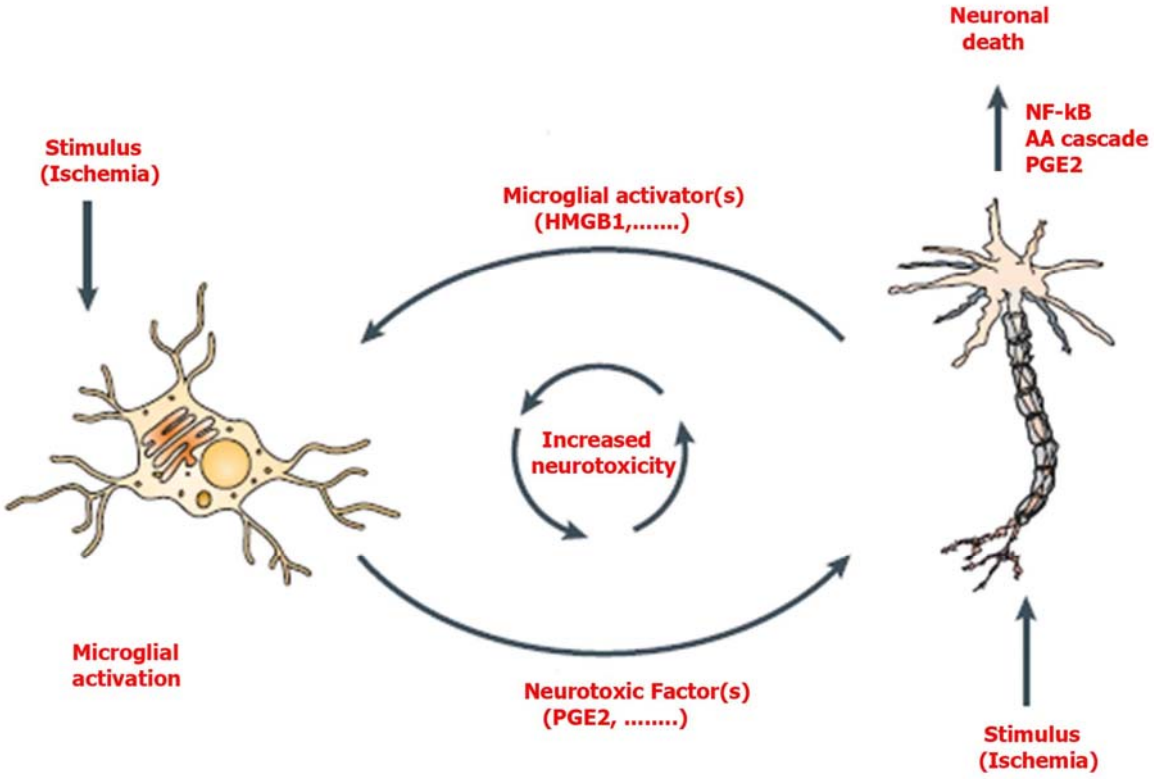


Figure 4.1. Suggested model for the interaction between neurons and microglia after insults such as ischemia, after M. L. Block et al., 2007 (with modification).

Abbreviation

4-HHE	4-hydroxyhexenal
4-HNE	4-hydroxynonenal
AA	Arachidonic acid
ABTS	2,2-azino-di-3-ethylbenzthiazoline-sulfonic acid
AGEs	advanced glycation end products
AP-1	Activator protein-1
ATF-2	Activating transcription factor-2
Aβ	amyloid β-peptide
BBB	Blood-brain barrier
BSA	Bovine serum albumin
C/EBPβ	CCAAT/enhancer binding protein (C/EBP) beta
Ca^{2+}	calcium
CD11b	Cluster of Differentiation molecule 11b
COX-2	Cyclooxygenase-2
cPLA-2	Cytosolic phospholipase A-2
DAPI	4′,6-Diamidino-2-phenylindole dihyd-rochloride
DCs	dendritic cells
DHA	Docosahexaenoic acid
DIV	day *in vitro*
DMEM	Dubellco's modified eagles medium
E16	embryonic day 16
Egr-1	Early growth response-1
eNOS	Endothelial nitric oxide synthase
EPOX	Epoxygenase
FBS	Fetal bovine serum
FDA	Food and drug administration
FFA	Free fatty acid
GFAP	Glial Fibrillary Acidic Protein
HBSS	Hank's balanced salt solution
HIF-1	Hypoxia inducible factor-1
HMGB1	High mobility group box 1 protein
Iba-1	Ionized calcium binding adaptor molecule 1
ICAM	Intracellular adhesion molecule-1
IFN-γ	Interferon-γ

IHC	Immunohistochemistry
IL-1ra	Interleukin-1 receptor antagonist
IL-1β	Interleukin-1 beta
iNOS	Inducible nitric oxide synthase
LB	Lysogeny broth Medium
LDH	lactate dehydrogenase
LOX	Lipoxygenases
LPS	Lipopolysaccharide
LysoPlsEtn	ethanolamine lysoplasmalogen
LysoPtdCho	lysophosphatidylcholine
MCAO	Middle cerebral artery occlusion
MCP-1	Monocyte chemotactic protein-1
MIP-1	Macrophage inflammatory protein-1 alpha
MMPs	Matrix metalloproteinases
Mn-SOD	manganese superoxide dismutase
mPGES-1	Microsomal prostaglandin E_2 synthase-1
NeuN	Neuronal Nuclei
NF-κB	Nuclear factor kappa B
NGS	Normal goat serum
NHS	Normal horse serum
NID	Non ionic detergent buffer
NLS	Nuclear localization sequence
NMRI	Naval Medical Research Institute
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
OGD	Oxygen glucose deprivation
P2	postnatal day 2
PAF	Platelet-activating factor
PARP	poly(ADP)-ribose polymerase enzyme
PBS	Phosphate balanced saline
PCR	Polymerase chain reaction
PFA	Para–formaldehyde
PGE_2	Prostaglandin E_2
Pgg_2	prostaglandin G_2
Pgh_2	prostaglandin H_2
PlsEtn	ethanolamine plasmalogen
PlsEtn-PLA2	Plasmalogen-selective phospholipase A2
PM	plasma membrane

PPAR-γ	Peroxisome proliferator-activated receptor
PtdCho	phosphatidylcholine
RAGE	Receptor for advanced glycation end products
RHD	Rel homology domain
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-PCR
STAT3	Signal transducer and activator of transcription 3
TG	transgene
TGF-β	Transforming growth factor-ß
TLR-2	Toll like receptor-2
TLR-4	Toll like receptor-4
TNF	Tumor necrosis factor
t-PA	Tissue plasminogen activator
VCAM	Vascular cell adhesion molecule-1
WT	wild-type

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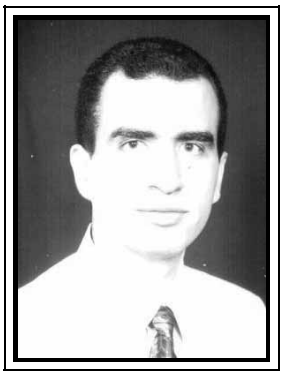
**S.M. and W.B. contributed equally to this work.*

2- Waleed Barakat, Anne Regnier-Vigouroux and Markus Schwaninger

"Regulation of prostaglandin E_2 release in cerebral ischemia"

In preparation.

Curriculum Vitae



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